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Morphology and histochemistry of the peripheral olfactory organ and behavioural responses by the round goby, Neogobius melanostomus , to putative pheromones and conspecific extracts.

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MORPHOLOGY AND HISTOCHEMISTRY OF THE PERIPHERAL OLFACTORY ORGAN AND BEHAVIOURAL RESPONSES BY THE ROUND GOBY, *NEOGOBIUS MELANOSTOMUS*, TO PUTATIVE PHEROMONES AND CONSPECIFIC EXTRACTS

By

Rachelle Marie Belanger

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ABSTRACT

This study links the anatomy of the peripheral olfactory organ in the bottom-dwelling round goby (*Neogobius melanostomus*) with behavioural responses to putative pheromones and conspecific extracts.

The spatial organization of the nasal cavity was examined using impression material injection, immunocytochemistry, and transmission electron microscopy. The peripheral olfactory organ had a compact structure with an olfactory chamber that contained a single longitudinal lamella. Prominent dorsocaudal lachrymal and ethmoid accessory nasal sacs were situated ventrocaudal to the chamber. The location of the olfactory mucosa within the olfactory chamber is novel for teleost fish, as it extends dorsally beyond the ventral surface. Microvillar and ciliated olfactory sensory neurons were identified by transmission electron microscopy. G_{olf}- immunoreactive ciliated olfactory sensory neurons and G_{o}- immunoreactive microvillar olfactory sensory neurons were located throughout the olfactory epithelium. G_{o}- immunoreactive crypt cells were also found throughout the olfactory epithelium of some specimens.

The presence of accessory nasal sacs indicates that the round goby may regulate water flow over the olfactory sensory surface through gill ventilation. Gill ventilation rates (opercular beats) were recorded from male and female, osmic and anosmic round gobies during exposure to the putative pheromones estrone and etiocholanolone (10^{-8} to 10^{-12} M). Osmic male round gobies demonstrated a significant ($p<0.05$) increase in basal ventilation to concentrations of estrone (10^{-8} and 10^{-9} M) in the winter (September, 2001 -

January, 2002), but responded to a greater range of concentrations (10^{-8} to 10^{-11} M) in the summer (May - June, 2002). Osmic females did not respond to etiocholanolone in the winter, but showed significant increases in basal ventilation in the summer to concentrations of 10^{-8} and 10^{-10} M.

The effect of female conspecific urogenital extracts on ventilation was studied on male round gobies. Osmic and anosmic male round gobies were exposed to urogenital extracts of females injected with human chorionic gonadotropin in the winter and summer. Osmic male round gobies showed no response to these extracts during the winter months, but demonstrated a significant response to urogenital extracts during the summer. This suggests that the round goby has evolved a complex intraspecific communication system which is mediated through olfaction.

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LIST OF ABBREVIATIONS

®	Registered
AN	Anterior naris
ANS	Accessory nasal sac(s)
AT	Acetylated tubulin
CNS	Central nervous system
$CuSO_4$	Copper sulfate
E1	Estrone
EOG	Electro-olfactogram
ETIO	Etiocholanolone
ETIO-g	etiocholanolone-glucuronide
G_0	G-Protein found in microvillar olfactory sensory neurons
$G_{\alpha 0}$	Designating the α subunit of the G-protein mentioned above
G_{olf}	G-Protein found in ciliated olfactory sensory neurons
$G_{\alpha olf}$	Designating the α subunit of the G-protein mentioned above
GnRH	Gonadotropin releasing hormone
GSI	Gonadosomatic index
HCG	Human Chorionic Gonadotropin
HSI	Hepatosomatic index
ICC	Immunocytochemistry
IR	Immunoreactive/ Immunoreactivity
M	Molar (moles/L)
MS-222	Tricaine methane sulphonate
NIS	Non-indigenous species
No.	Number
OC	Olfactory chamber
OSN(s)	Olfactory sensory neuron(s)
PN	Posterior naris
ppm	Parts per million or mg/L
POO	Peripheral olfactory organ
S.E.	Standard Error
SFW	Summer fish weight
SGSI	Summer gonadosomatic index
SHSI	Summer hepatosomatic index
Summer	May to June 2002
TEM	Transmission electron microscopy
TL	Total length
WFW	Winter fish weight
WGSI	Winter gonadosomatic index
WHSI	Winter hepatosomatic index
Winter	September 2001 to January 2002

GENERAL INTRODUCTION

Olfaction exerts a functional role in many aspects of the reproductive process, and considerable work on the role of chemical signals and pheromones in the reproductive behaviour of fishes has been conducted. A relationship between endocrine activity and sex pheromone function in fish was first in established studies of a goby (*Bathygobius soporator*) (Tavolga 1955, 1956). Studies on crab pheromones led to the first clear indication that aquatic organisms might commonly use released hormone metabolites as pheromones (Kittredge *et al.* 1971).

Pheromones have been defined as substances that are secreted to the outside by an individual and received by a second individual of the same species, which releases a specific reaction such as a definite behaviour or a developmental process (Karlson and Lüscher 1959). These authors also added that pheromones, as chemical messengers, should be active in minute amounts and be relatively species-specific, only limited molecular overlapping between closely-related species being tolerated.

Kittredge and Takahashi (1972) speculated that hormone metabolites are likely to function as pheromones in many species of aquatic animals as they would have evolved the chemosensory mechanisms to detect sexually receptive conspecifics. Reproductive behaviour and olfactory anatomy and physiology of the pheromone receiver should then be synchronized with a particular physiological event, increasing the chance of reproductive success in that species. Additional research has shown that hormonally derived chemical signals (hormonal pheromones) are also used by many fish including

fish from the family Gobiidae, Cottidae, Clariidae, and Cyprinidae (Colombo *et al.* 1982; Døving 1976; Sorensen *et al.* 1988; Stacey and Sorensen 1991; Sorensen 1992; Stacey *et al.* 1994a). Nevertheless, the chemical nature and function of pheromones is still poorly understood (Stacey and Sorensen 1991).

Fluids or swabs of the urogenital area, presumed to contain sex pheromones, of gobiid conspecifics, have evoked responses using physiological and/or behavioural measures (e.g. Tavolga 1956; Colombo *et al.* 1982). Moreover, electrophysiological studies indicate that responses to pheromones are mediated through olfaction (Zippel *et al.* 1997; Eisthen 1992; Sorensen 2001).

Knowledge of the specificity of pheromones used during reproduction may aid biologists and conservation biologists in manipulating the breeding seasons of fish. In some cases, pheromone manipulation of fish behaviour and physiology may be a cost effective, time saving, species specific method to control for exotic pest species. Pheromone research has previously been used to control insect pests of agricultural crops (review by Shani 2000), a method which is currently being researched for the population control of sea lamprey, *Petromyzon marinus* (Li *et al.* 2002).

It has been estimated that fish olfactory systems can distinguish about a hundred individual chemical stimulants (review by Sorensen and Caprio 1998). Little is known about the chemosensory abilities of most fish species, some of which most likely have different chemical sensitivities and specificities because of the varied nature of their behavioural ecology and taxonomy (review by Sorensen and Caprio 1998).

The objective of my research was to relate the structure of the peripheral olfactory

organ with behaviour in the non-indigenous round goby, *Neogobius melanostomus*. Murphy *et al.* (2001) have shown that both male and female round gobies respond physiologically (through electo-olfactogram readings) and behaviourally (increasing basal ventilation) to various putative pheromones. Their data have provided an experimental basis for testing involvement of pheromonal communication during reproduction. Murphy's (1998) study provided the framework for my research on identifying the structure of the peripheral olfactory organ in the round goby and its function in reproductive behaviour. Knowledge of the structure of the olfactory system in the round goby is important for electrophysiology and deprivation. Olfactory organ structure has not been examined in any gobiid species, even thought they are the largest family of marine teleosts. Sex pheromone testing in the round goby is novel, as this is the first study to examine behaviours of the round goby following exposure to urogenital extracts.

Biology of the round goby:

The round goby, *Neogobius melanostomus* (Pallas 1811) (Perciformes: Gobiidae) is a bottom-dwelling fish of the family Gobiidae (subfamily: Neogobiinae) and is native to the Black, Caspian, Marmara, Azov and Aral seas of the Ponto-Caspian region (Simonović *et al.* 1996). Members of the genus *Neogobius* have fused pelvic fins and elongated dorsal and anal fins (Miller 1986) and are distinguished from the Atlantic and Mediterranean basin *Gobius* spp. by the absence of a swimbladder and location of the uppermost rays of the pectoral fins within the fin membrane (Pinchuk 1991). Characteristics of the round goby are its brown or mottled colouration, fused pelvic fin,

blunt snout, bulging eyes, black dot on the dorsal fin, and an overall round appearance. The round goby can attain a maximum length of 300 mm with the average size of the males being 120 mm and the 70 mm for the females (Charlebois *et al.* 1997).

In its native region, juvenile round gobies feed on benthic invertebrates while adult round gobies feed particularly on bivalve mollusks as they have molariform pharyngeal teeth which allow them to crush hard prey (French 1993; Ghedotti *et al.* 1995). In the Danube River, a tributary to the Black Sea, the major prey of the round goby is the zebra mussel, *Dreissena polymorpha* (Simonović *et al.* 2001). The life-span of the round goby is four years but some individuals live as long as 7 or 8 years (Miller 1984). Male round gobies mature primarily at age three, whereas females generally mature at age two and are smaller than males (Miller 1984; 1986). Male round gobies grow at a faster rate than females after the first year of life, but within a cohort, individuals will exhibit different growth strategies (Bil'ko 1971). After attaining a large size, it is thought that males spawn once and then die (Berg 1949). Round gobies are typically reported at depths ranging from 0.2 to 2m (Charlebois *et al.* 1997), but have been found in Lake Erie at depths of 7-11m (Wickett and Corkum 1998) and 28m in Lake Michigan with similar depths in Lake Huron (J. Janssen, personal communication).

Like other species of gobiids, round goby males occupy, maintain and defend nests against intrusion by predators. Males migrate onto spawning grounds in the early spring and set up territories prior to the arrival of the females (Kovtun 1980). Adult male round gobies are characterized by their large size at maturity, enlarged cheeks and overall charcoal-black colouration when breeding (Nikol'skii 1963; Miller 1984). Females enter

these nests and deposit eggs: the male fertilizes and maintains the eggs and nest for 28 days at 14°C (Miller 1984; MacInnis and Corkum 2000; J. Janssen and L. Corkum, personal communication). Nests are typically found at depths of 0.5 to 2 m and under rocks or logs, in beer cans, or in a structure with one opening (Charlebois *et al.* 1997; Jude 1997; Wickett and Corkum 1998). In the Great Lakes, the spawning period extends from May to August and overlaps with the spawning of native fish (Charlebois *et al.* 1997; Corkum *et al.* 1998). Simonović *et al.* 2001, report that by late September (Danube River, Yugoslavia), the gonads of male and female round gobies are small and firmly attached to the dorsal wall of the body cavity, indicating the end of the reproductive season.

Eggs of the female round gobies are demersal adhesive and are typically spawned on any hard overhead surface in a nest which is guarded against predators by the male. The cylindrical/cone-shaped eggs are arranged in a single layer, enabling the male to aerate a compact patch of eggs efficiently (Corkum *et al.* 1998; Wickett and Corkum 1998). The hanging egg position and characteristic shape aid in cleansing since water will flow around the egg surfaces and sediment particles are easily shed (Miller 1984). Fertilization rates in the round goby may be as high as 95% and a male can hatch up to 95% of the eggs in his nest (Charlebois *et al.* 1997). These factors give the round goby the potential to dominate nesting sites and produce large numbers of offspring each year (Corkum *et al.* 1998).

Range and Impacts of round gobies in the Great Lakes:

The round goby is a nonindigenous species (NIS) and was first discovered in the Great Lakes in June 1990 (Crossman *et al.* 1992; Jude *et al.* 1992). NIS are successfully reproducing organisms transported by humans into an area outside their historic or geographic range (Fuller *et al.* 1999). Within five years of their discovery in the St. Clair River, round goby populations had spread to all five Great Lakes (Jude 1997). They are now also found in several tributaries in Michigan (Flint, Shiawassee, and Saginaw Rivers), Ontario (Running Creek, Sydham and Napanee Rivers), and in the Chicago Sanitary and Shipping Canal en route to the Mississippi River (Steingraeber *et al.* 1996; Steingraeber and Thiel 2000). Initial introduction to North America was likely through the ballast water of foreign vessels presumed to have originated from the Ponto-Caspian region (Jude *et al.* 1992). Initial round goby catches in the Great Lakes were in harbour locations, as they were likely dispersed rapidly in the Great Lakes by freighters moving from port to port (Jude 1997; Ray and Corkum 2001).

The round goby has been able to spread and proliferate in Great Lakes habitats for a number reasons and has several characteristics of successful colonizers. It tolerates a wide range of environmental conditions; has a diverse diet that includes dreissenids, soft-bodied invertebrates and fish; has a large body size compared with species of similar benthic lifestyle; feeds nocturnally; behaves aggressively; spawns repeatedly throughout spring and summer; and facilitates successful larval recruitment through parental care (Leach 1995; Ray and Corkum 1997; MacInnis and Corkum 2000; Charlebois *et al.* 1997, 2001; Jude 2001). Round gobies have the ability to attain high abundances in optimal,

rocky substrate area in the face of native fish communities (Jude 1997) and are large enough (often > 150 mm) that they are a nuisance to anglers who frequently give up fishing for sport fish because of bait wastage (J. Janssen, personal communication).

Estimating the environmental and economic costs of NIS in the Great Lakes is difficult, nevertheless, it is known that damage and losses associated with exotics total approximately $137 billion US per year (Pimentel *et al.* 2000). Approximately ten percent of the Great Lakes' NIS have had significant influences, both economic and ecological. It is known that introduced fishes frequently alter food webs of aquatic ecosystems (Pimentel *et al.* 2000). As round goby populations increase in the Great Lakes, their effects on indigenous species are becoming evident. There is concern about the potential impact of the round goby on native fish species as the round goby may have a competitive advantage over native mottled sculpins and logperch for food resources in the St. Clair River (French and Jude 2001). Janssen and Jude (2001) documented a decline of the native mottled sculpin (*Cottus bairdi*) in southern Lake Michigan after the introduction of the round goby. Both species are benthic with similar ecological requirements for nesting, feeding, and shelter but the ultimate recruitment failure and subsequent demise of the mottled sculpin most likely were caused by spawning interference by the round goby (Dubs and Corkum 1996; Charlebois *et al.* 2001; Janssen and Jude 2001).

Population densities of round gobies in the St. Clair River have increased exponentially in the last 10 years. This species now comprises more than 50% of the catch in the lower part of the St. Clair River (Nichols *et al.* 1999). Unfortunately, one of

the few known spawning grounds of lake sturgeon occurs in lower St. Clair River. The spawning reef is heavily colonized by round gobies, with densities as high as 25/m^2. The predaceous behaviour exhibited by round gobies combined with such high population densities, raises questions about their impairment to lake sturgeon recruitment. Round goby populations increase from 10/m^2 to 25/m^2 after sturgeon have deposited their eggs. Sturgeon egg densities are known to decline from 2554/m^2 as adult sturgeon leave the reef to an average of 185 egg/m^2 48 hours later (Nichols *et al.* 1999). This, along with gut analysis suggests that round gobies and native fish are ingesting sturgeon eggs (Nichols *et al.* 1999). The similar predation of lake trout (*Salvelinus namaycush*), smallmouth bass, and mottled sculpin eggs by high densities of round gobies at some Great Lakes sites leads to the prediction that the round goby may negatively affect other native fishes reproduction and rehabilitation (Chotkowski and Marsden 1999; Janssen and Jude 2001; J. Janssen and G. Steinhart, personal communication). Charlebois *et al.* (2001) suggested that natural chemical control through pheromonal attraction mediated by olfaction be investigated as a plausible means of controlling round goby populations in areas where native fishes spawn, to lessen or remedy the impacts they are having on the Great Lakes commercial and recreational fisheries.

Olfaction and odour signaling:

From bacteria to mammals, detecting chemicals in the environment has been critical to the success of organisms. As much as 4% of the genome is dedicated to olfactory sensory neurons (OSNs) and olfactory processing in vertebrates (Firestein

2001). Among higher eukaryotes, there is an evolutionary convergence towards a conserved organization of signaling pathways in olfactory systems (Hildebrand and Shephard 1997). The main olfactory system is the primary sense used to find food, detect predators and prey, and mark territory. A second olfactory sense, has developed for the specific task of finding a receptive mate, a task of sufficient complexity. This system specializes in recognizing pheromones which contain information about location, reproductive state, and availability of a particular species (Firestein 2001). Thus olfactory ability and the function of this second olfactory system in the round goby are the focus of this study.

In fish, odour molecules enter the nasal cavity via the anterior nostril, where they stimulate OSNs expressing a particular receptor. The axons of these neurons make synaptic connections with the second order neurons and mitral cells located within glomeruli of the olfactory bulb (reviewed by Zielinski and Hara 2001; Mombaerts *et al.* 1996) (Figure 1). Fish have two types of OSNs, ciliated and microvillous. In these sensory neurons, cilia or microvilli extend from the apical surface of the dendrite into the lumen of the nasal cavity. Ciliated cells have up to 8 cilia, each up to 10 μm long, which project laterally from their olfactory knob (Sorensen and Caprio 1998). Microvillous cells have a rounded surface, from which up to 80 microvilli up to 5 μm emanate (Zeiske *et al.* 1992). The cell body of ciliated OSNs is located in the lower third of the olfactory epithelium, making the dendrite longer than the shorter dendrite of microvillar OSNs. The cell body of the microvillar OSNs is in the upper third of the olfactory epithelium (Morita and Finger 1998).

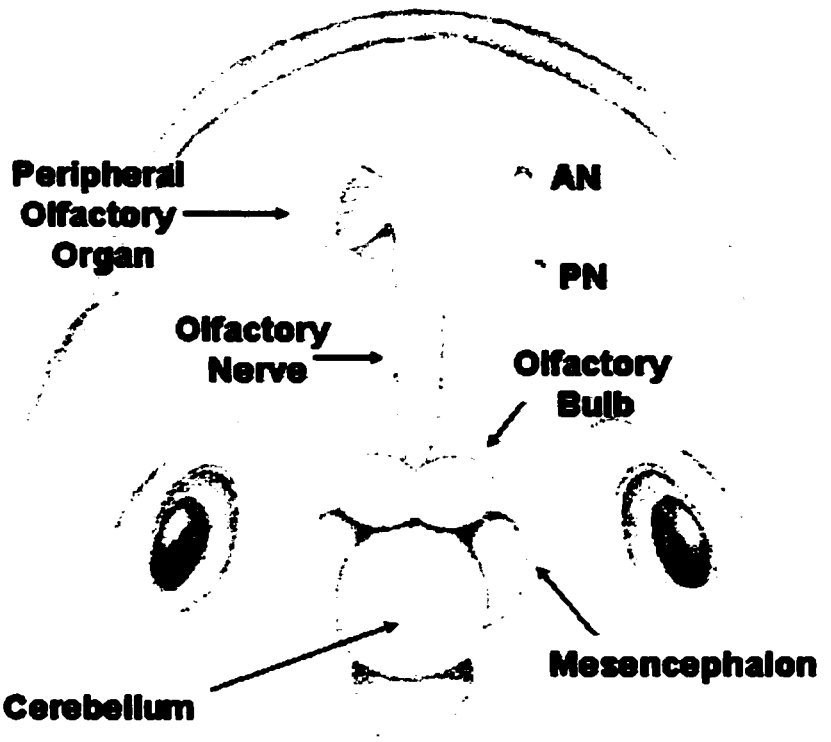

Figure 1: A pictural representation of the olfactory system in teleosts from the peripheral olfactory organ, located between the anterior naris (AN) and posterior naris (PN). The peripheral olfactory organ contains olfactory epithelium with sensory neurons. The olfactory nerve connects the peripheral olfactory organ with the olfactory bulb. Figure modified from Belanger *et al. in press* and Hara 1975.

OSN dimorphism may be related to odourant quality distinction although the functional distinction between teleost microvillar and ciliated OSNs is yet to be clearly defined. Indirect evidence shows that ciliated OSNs respond to bile acids in salmonids, amino acids (Tommesen 1983), bile acids in rainbow trout (Zielinski and Hara 1988), and amino acids in goldfish (Zippel *et al.* 1997). Work on goldfish by Zippel *et al.* (1997)

suggests that microvillar OSNs mediate responses to pheromones. Retrograde labelling performed on catfish (Hansen *et al.* 2002) and goldfish (Sorensen *et al.* 2002) suggests that microvillar, as well as crypt cells perceive pheromones. Eisthen (1992) also suggests that the presence of microvillar OSNs in the mammalian vomeronasal organ, which is believed to receive pheromone stimulation, are responsible for perception of pheromones.

How individual vertebrate OSNs transduce receptor binding events into electrical impulses has been subject of much study as fish have been important models in these investigations. In fish, olfactory signaling responses are mediated by approximately 5 to 10 million OSNs per nostril (Yamamoto 1982), each containing heterotrimeric GTP-binding proteins (G-proteins) (Bruch *et al.* 1989; Sosinsky *et al.* 2000). Most odour molecules are recognized by more than one receptor cells and most receptors cells recognize several odours, probably related by chemical property (review by Firestein 2001). It presently appears that as in other vertebrates, the G-proteins found on fish OSNs likely exert their actions through a second-messengers system, an adenylate cyclase system. Antibodies against the G-protein G_{olf} have been used to demonstrate that these G-proteins are found in catfish olfactory cilia (Abogadie *et al.* 1995; Huque and Bruch 1986) and G_{o} immunoreactive proteins are located in vomeronasal sensory neurons in terrestrial vertebrates (Matsuoka *et al.* 2001).

When an odour enters the olfactory chamber it binds the extracellular surface G-protein coupled receptor. Following this, a cascade of events is initiated that transforms the chemical energy of binding into a neuronal signal, changing the membrane potential (review by Firestein 2001). The ligand-bound receptor activates the G-protein (G_{olf})

(Jones and Reed 1989) and the alpha subunit of the G-protein detaches and in turn attaches to and activates adenylate cyclase (Pace and Lancet 1986). Once this occurs, abundant ATP is converted to cyclic AMP, a signaling molecule. Cyclic AMP then binds to the intracelluar surface of an ion channel (a cyclic nucleotide-gated channel) and the channel opens, allowing an influx of Ca^{2+} and Na^{+} (Firestein *et al.* 1991) (Figure 2). Schlid and Restrepo (1998) and Liu *et al.* (1999) have shown that OSNs expressing the G_0 may have a different olfactory signaling cascade where the alpha subunit of G_0 binds to the phospholipase C. The second messenger inositol triphosphate (IP_3) binds to and actives a calcium channel. Inactive OSNs normally maintain a resting voltage of about -65 mV, but when the cyclic nucleotide-gated channels open, the influx of and Na^{+} and Ca^{2+} ions causes the inside of the cell to become less negative. If the channels are open for long enough, this causes the membrane potential to become about 20 mV less negative and the cell reaches threshold and generates an action potential. The action potential is then propagated along the OSN axon to the olfactory bulb (review by Firestein 2001).

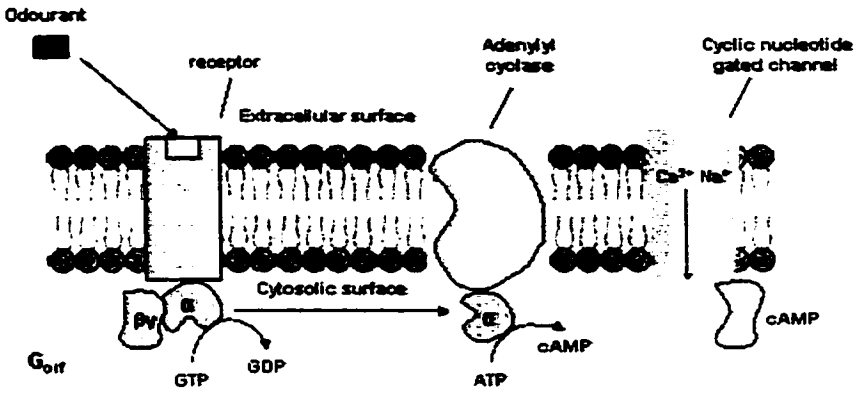

Figure 2: Model representing the mechanism of odourant signal transduction in olfactory sensory neurons. The chemical energy following the binding of an odourant to a G-protein coupled receptor, explained in the text, is transformed to an electrical stimulus by a cascade of enzymatic reactions. This diagram represents a pathways using the second messenger cAMP. Diagram by Rachelle Belanger.

There are several physiological mechanisms that OSNs use for adjusting the OSN sensitivity to odourants. A negative feedback pathway is important for response adaptation (Kurahashi and Menini 1997). As intracellular calcium increases during the odour response, it acts on the channel to decreases it sensitivity to cAMP, thereby requiring a stronger odour stimulus to produce sufficient cAMP to open the channel (Chen and Yau 1994; Liu *et al.* 1994; Kramer and Siegelbaum1992). Other mechanisms for adjusting sensitivity includes a regulator of G-protein signaling which acts on the adenylyl cyclase to decrease its sensitivity (Sinnarajah *et al.* 2001), and protein kinase A that phosphorylates activated receptors sending them into a desensitized state (Dawson *et*

al. 1993; Fadool and Ache 1992).

Thesis Overview:

The purpose of this study was to determine the spatial organization of the peripheral olfactory organ and to link the peripheral olfactory organ anatomy to behavioural responses to putative pheromones and gonadal extracts in non-indigenous round gobies collected from the Detroit River and the western basin of Lake Erie. The overall goal of this research is to decrease the reproductive potential of the round goby by using conspecific urogenital extracts as an attractant. This may be used to attract round gobies away from habitats where predation by this species threatens native fish populations.

Chapter one presents information on the gross and fine structure of the peripheral olfactory organ in the round goby. Knowledge of the cellular composition and organization of the peripheral olfactory organ is essential for physiological analysis of olfactory responses. Dental impression material, immunocytochemistry using antibodies against acetylated tubulin, $G_{\alpha o}$, and $G_{\alpha olf}$, and transmission electron microscopy were effective techniques used to determine the gross and fine structure, including the spatial arrangement of microvillar and ciliated OSNs in the peripheral olfactory organ. Gross structure analysis allowed me to conclude that there is a link between olfaction and behaviour. The discovery of lachrymal and ethmoidal accessory nasal sacs implies that these propel water through the nares at a controlled rate. Moreover, the rate may be controlled through gill ventilation (opercular beats).

The second chapter presents the results of behavioural bioassays focusing on gill ventilation rates of osmic and anosmic, male and female (gravid and non-gravid) round gobies exposed to 10^{-8} to 10^{-12} M concentrations of the putative pheromones estrone and ethiocholanolone respectively. Responses to these compounds by osmic round gobies were determined and compared to responses from tissue extracts of gravid females and non-gravid females, both injected with human chorionic gonadotropin (HCG). Data on copper sulfate olfactory sensory deprivation, occlusion of one nares and changes in basal ventilation rates were investigated for control purposes and to determine if olfactory sensory input was essential for the gill ventilation responses. The second part of chapter two presents data on the physical characteristics of the fish used in the study, such as total length of the fish, weight, gonadosomatic, and hepatosomatic indices to examine the sexual maturity and fitness of the round gobies.

This study was conducted to answer questions of pheromonal communication in round gobies. This will lead to control of round gobies in areas where native fish spawn as eradication of established introductions is usually impossible (Leach 1995). Pheromonal techniques have been used successfully to control and monitor a variety of insect pests (review by Shani 2000). Other studies have shown that natural pheromones can also be used as attractants in aquatic species (e.g., sea lamprey by Teeter 1980; Bjerselius *et al.* 2000; Li *et al.*, 2002). The anatomical studies and behavioural bioassays will help characterize the contribution of pheromones to goby reproduction. Also, my research may eventually lead to the development of a technology for managing goby populations in habitats where the species co-occurs with spawning sport and commercial

fishes. Development of a pheromone-based strategy may be used to disrupt the reproductive habitats of the round gobies in their known nursery areas, reefs and shipwrecks. This approach can also be used in spawning areas of native fishes (smallmouth bass and lake sturgeon) to prevent round gobies from preying on the eggs of these fish.

CHAPTER 1: Morphology and Histochemistry of the Peripheral Olfactory Organ in the Round Goby, *Neogobius melanostomus* (Teleostei: Gobiidae)

INTRODUCTION

The olfactory organ of fishes shows considerable diversity that reflects the degree of development and ecological habitats. The teleost peripheral olfactory organ is comprised of an anterior naris, olfactory chamber with OSNs, and a posterior naris. The OSNs located within the peripheral olfactory organ converge into nerve fascicles and synapse at the olfactory bulb, located in the forebrain (Firestein 2001). Unlike terrestrial vertebrates, the peripheral olfactory organ in teleosts is not directly responsible for respiration as there is no direct connection between the olfactory and respiratory systems. In many species, accessory nasal sacs assist with water ventilation through the nasal cavity (Burne 1909; Johnson and Brown 1962; Døving *et al.* 1977; Melinkat and Zeiske 1979). The nasal cavity of teleost fishes is relatively shallow and lined by a ciliated pseudostratified epithelium. Water enters through an anterior naris, flows over OSNs and leaves through the posterior naris.

Structure of the peripheral olfactory organ in fish:

During olfactory stimulation, soluble odourants enter the olfactory chamber through the anterior naris and flow over the surface of OSNs where the molecules bind to G protein coupled receptors located on the extracellular surface of the plasma membrane

covering of cilia and microvilli. Olfactory mucosa containing the OSNs are typically located on the floor of the olfactory chamber, which is often folded, forming olfactory lamellae (e.g., Kleerekoper 1969; Hara 1975). The extent of olfactory lamellar folding within the teleost superorder Acanthopterygii varies considerably, from a flat unfolded surface (e.g. *Rudarius arcodes*, *Ostracion tuberculatus*) to a multi-lamellar rosette with over 100 folds (e.g., morays, trout, and eels) (Yamamoto 1982). In the economically important order Perciformes, the number of olfactory lamellae varies from 0 (*Omobranchus elegans*) to 64 (*Upeneus bensasi*) (Yamamoto 1982). The large surface area provided by olfactory lamellae enables many OSNs to populate the relatively small teleost olfactory chamber. Fish with multi-lamellar peripheral olfactory organs have an acute sense of smell and various aspects of their life history, such as feeding and reproduction, are mediated through olfactory cues (Hara 1992). On the other hand, in teleosts with a small lamellar surface, OSNs may extend beyond the ventral surface of the nasal cavity. For example, olfactory epithelium extends to the lateral walls of the olfactory chamber of *Spinachia spinachia* (Perciformes; Gasterosteidae) (Theisen 1982). Olfactory epithelium has not been shown on the dorsal surface of the peripheral olfactory organ in any teleost. Some tetrapods, such as amphibians, contain a relatively flat nasal cavity, with olfactory epithelium on the ventral and dorsal surfaces (e.g. *Xenopus laevis*; Hansen *et al.* 1998).

The gobiid fishes (Perciformes: Gobiidae) are the most speciose of all suborders of bony fishes, with over 2500 nominal species arranged in at least 300 genera (Miller 1993). The peripheral olfactory organ is unilamellar, with a single fold along the rostro-

caudal axis in five species of the family Gobiidae (*Acanthogobius flavimanus, Chasmichthys dolichognathus, Sagamia geneionema, Chaenogobius urotaena, Odontamblyopus rubicundus*) (Yamamoto and Ueda 1979), however the olfactory organ of *Neogobius* has not been examined.

Olfactory Sensory Neurons (OSNs):

Two morphological types of OSNs, ciliated and microvillar, have been distinguished in the peripheral olfactory organ of teleost fish through identification of the cilia or microvilli extending from the apical olfactory knob on the OSN mucosal surface (e.g., Evans *et al.* 1982; Zielinski and Hara 1988; Hansen *et al.* 1999). A third sensory neuron, the crypt cell, is also present in several species (Hansen *et al.* 2001). Ciliated OSNs have a slender tall dendrite and perikaryon in the lower region of the olfactory epithelium, whereas the dendrites of microvillar OSNs are shorter, with the perikaryon in the superficial region of the olfactory epithelium (Morita and Finger 1998). However, the functional distinction between teleost microvillar and ciliated OSNs is yet to be clearly defined. The G protein, G_{olf} is expressed by OSNs in mammals (Jones and Reed 1989), amphibians (Mezler *et al.* 2001), and ciliated OSNs in teleosts (Abogadie *et al.* 1995). Sensory neurons in the terrestrial vertebrate chemical sensing organ, the vomeronasal organ, express the G protein $G_{\alpha o}$ (e.g., Jia and Halpern 1996) as do microvillar OSNs in catfish and goldfish (Hansen *et al.* 2001). Therefore, $G_{\alpha olf}$ immunoreactivity of ciliated OSNs and $G_{\alpha o}$ immunoreactivity of microvillar OSNs may be useful for determining if these cells extend beyond the ventral surface of the olfactory chamber in Gobiidae.

Antibodies against G proteins were used to show that G proteins are located in sensory neurons of the olfactory epithelium in terrestrial and aquatic vertebrates (Jia and Halpern 1996, Dellacorte *et al.* 1996, Abogradie *et al.* 1995, Shinohara *et al.* 1992, Mezler *et al.* 2001, Wekesa and Anholt 1999). This is the first study to use antibodies against G-proteins for mapping the distribution of OSNs in the peripheral olfactory organ.

Structurally, OSNs are bipolar primary neurons with a cylindrical dendrite that terminates at the apical surface of the epithelium and is directly exposed to the external environment. The distal end of the dendrite forms the large olfactory knob which protrudes slightly above the epithelial surface and the proximal part of the perikaryon tapers off to form an axon. The axons continue through the lamina propria and converge and form olfactory nerve fascicles, which run posteriorly and end in the olfactory bulb. The ciliated OSN generally contains 4 to 8 cilia which radiate from the olfactory knob. Usually the cilia show the 9+2 arrangement of microtubules. Microvilli on the surface of microvillar OSNs can number from 30 to 80 depending on the species, with the cytoplasm of microvillar OSNs containing similar ultrastructural (e.g. cell junctions and mitochondria) features as that of ciliated OSNs.

Accessory Nasal Sacs:

In some fish, the opening and closing of the mouth causes the pumping action of accessory nasal sacs, which aids in the flow of water through the olfactory chamber in the nasal cavity (Parker 1910; Pipping 1926; Lierman 1933; Melinkat and Zeiske 1979; Nevitt 1991). The occurrence of accessory sacs in fish has long been associated with

semi-sedentary life (Kyle 1899), and may be necessary for unidirectional water flow across the olfactory chamber in stationary bottom-dwelling fish (Burne 1909; Kapoor and Ojha 1972). Fish with accessory sacs for the pumping of water though the peripheral olfactory organ have previously been designated cyclosomates, and fish using only the beating of ciliated cells were termed isomates (Døving *et al.* 1977). In cyclosomates, ciliary beating and accessory sac ventilation likely have a co-operative role for moving odourant molecules over the olfactory epithelium. Cyclosomate teleosts have either one or two accessory nasal sacs that may either be linked or fused, such as those observed in Pleuronectiformes (Kyle 1899; Burne 1909; Chabanaud 1927; Lierman 1933; Kleerekoper 1969; Applebaum and Schemmel 1983; Webb 1993) or distinct, separate structures such as those found in many of the Perciformes (Burne 1909; Sinha and Sinha 1990). Species in two families of Perciformes, Trachinidae and Scombridae, contain single accessory nasal sacs (Burne 1909). When two accessory nasal sacs are present, they generally follow the ethmoidal and lachrymal bone structure of the skeletal system and are thus designated the ethmoidal and lachrymal sacs after Burne's (1909) nomenclature or the dorsomesial and ventromesial sacs (Kapoor and Ojha 1973). In *Spinachia spinachia* (Gasterosteidae), the lachrymal sac was larger than the ethmoidal sac and both sacs were lined with nonsensory epithelium (Theisen 1982). There have been no studies on the occurrence of accessory nasal sacs in fish belonging to the Gobiidae family.

Olfaction and reproductive behaviour of gobies:

The round goby displays reproductive behaviour that may be mediated through intra-specific communication by pheromones. For example, the male occupies a nest chamber, defends it against intrusion by predators, and allows gravid females to enter for spawning (Miller 1984; MacInnis and Corkum 2000). Physiological studies have shown that steroidal putative pheromones evoke electro-olfactogram responses from the anterior region of the nasal cavity (Murphy *et al.* 2001). However, further physiological and behavioral studies have been hampered by lack of information on the spatial organization of the nasal cavity in gobies. Therefore, the objective of this study was to determine the morphology and spatial organization of the peripheral olfactory organ in the round goby.

The reproductive success of the round goby may be a consequence of pheromonal communication related spawning behaviour. Experiments on the frillfin goby (*Bathygobius soporator*) showed that gravid females and extracts of the mature female goby urogenital system attracted mature males and caused them to exhibit reproductive behaviours (Tavolga 1956). Over 40 years later, the findings of C. Murphy (1998) suggested that steroidal compounds function as reproductive pheromones in the round goby. Two estrones, 17ß-estradiol-3ß-glucuronide, and etiocholanolone, elicited electro-olfactogram responses and gill ventilation rate increases in sexually mature male round gobies (Murphy *et al.* 2001).

Study Objectives:

One of the constraining factors in the study of pheromonally mediated reproductive behaviour in the goby is the lack of information available on its olfactory system. Knowledge of the cellular composition and organization of the peripheral olfactory organ is essential for physiological analysis of olfactory responses. Surprisingly, there are no published accounts of the cellular make up of the peripheral olfactory organ in any species of goby, although Gobiidae is the most speciose family of marine teleosts. The objective of this study is to determine the morphology and fine structure of the peripheral olfactory organ in the round goby, *Neogobius melanstomus* (Perciformes: Gobiidae). It is important to understand the organization of the peripheral olfactory organ in order to relate structure with function and to enable total olfactory sensory deprivation during ensuing behavioural tests.

I hypothesized that the round goby olfactory epithelium would contain both ciliated and microvillar OSNs, there would be no difference in the number or spatial arrangement of these OSNs, and that olfactory epithelium would be limited to the ventral floor of the olfactory chamber. It was expected that the OSNs would have a continuous, even distribution because Holl (1965) showed that in other fish species, the olfactory epithelium was continuous except for the dorsal parts of the epithelium in *Ictalurus, Anguilla, Perca, Salmo* and others. Murphy *et al.* (2001) showed that the round goby responded to odourants at very low concentrations (10^{-10} M), so it is expected that the peripheral olfactory organ is highly organized and inundated with OSNs.

In this study, the organization of the round goby nasal cavity, including the accessory sacs, was examined through the use of vinyl polysiloxane impression material. Acetylated tubulin immunocytochemistry was used to examine the extent of ciliation in the nasal cavity and the location of the OSNs. Ciliated and microvillar OSNs were identified by transmission electron microscopy and the spatial distribution of these cells was determined through immunocytochemistry against the G proteins, $G_{\alpha olf}$ and $G_{\alpha o}$. It is important to determine the spatial organization of the peripheral olfactory organ to render the round goby anosmic for behavioral studies. Understanding the structural organization will help with localizing OSNs in the future for examining physiological responses to pheromones.

MATERIALS AND METHODS

Round Goby Collections and Housing:

Neogobius melanostomus (Pallas 1811) male adults were collected from the Detroit River between August, 2000 and August, 2001 by angling in the Detroit River (Windsor, Ontario, Canada) and by trawling in the Canadian waters of the western basin of Lake Erie. Round gobies were kept in aerated coolers for transport back to the lab and held overnight in an aerated transportation coolers. Afterwards, the round gobies were sexed by examination of the genital papilla (Miller 1984). Males and females were maintained separately in holding tanks with a flow-through dechlorinated tap water

system (10-20°C) and a 16L:8D photoperiod. Round goby diets consisted of Nutrafin® fish flakes and zebra mussels (*Dreissena polymorpha*) when available. Tanks contained fine aquarium gravel, an air stone, and PVC shelters. All experimental procedures reported in this study were in compliance with guidelines established by the Canadian Council of Animal Care.

Nasal Molds:

Reprosil® (626170. Dentsply International Inc., Milford, DE), hydrophilic vinyl polysiloxane impression material, was used to make solid molds of the round goby peripheral olfactory organ to determine its gross structure. The orange base was initially diluted with acetone and the excess was drained off before adding the base to the solidifier. After the base and the solidifier were mixed, it was drawn into a 3 cc syringe and the tip was wiped clean before a 23-gauge needle was screwed on. This enabled injection of the, still liquid, impression material into the posterior nostril of a round goby heavily anesthetized with tricaine methane sulphonate (MS-222; Finquel, Fisheries Chemical Division, Redmond, Washington). The impression material was allowed to polymerize within the nasal cavity for 10 minutes. The dorsal skin layers were then removed by dissection and the mold was carefully removed from the nasal cavity and viewed using a dissecting microscope.

Solutions and Staining Procedures:

Recipes of all solutions and staining procedures are given in the Appendix A.

Fixation of the olfactory organ

Round gobies were deeply anaesthetized in MS-222 in preparation for cardiac perfusion. Before the cardiac perfusion, Zamboni's fixative was injected into both nostrils of the round goby. In some cases, 4% paraformaldehyde was used instead of Zamboni's fixative. The goby was placed on its dorsal surface on a Styrofoam base and an ventral incision was made caudal to the gills. The heart cavity was exposed by holding the animal open using needles stuck into the Styrofoam and care was taken to remove the pericardium. A 24 gauge butterfly needle (Ingram's Medical Supplier, Windsor, Ontario) was inserted into the ventricles of the heart and was clamped using a micro-haemostat between the ventricles and the cone. Once the needle was secure in the heart, 6.3% saline was dripped into the heart using an intravenous unit. At this time, the ventricles of the heart were cut to allow the blood from the body to pool in the cavity. The blood was withdrawn from the heart cavity using a disposable pasteur pipette.

Once the liquid filling the chest cavity became clear (i.e. most of the blood had left the body), then the intravenous unit was then switched to Zamboni's fixative. Once this began, muscle contractions began in the body of the round goby and the animal had to be held down in order to keep the needle from puncturing the heart. Once the contractions stopped, the round goby was left with Zamboni's fixative dripping into it for 20-30 minutes, or until the tissues appeared yellow and the tail was stiff. In some cases, paraformaldehyde and Zamboni's fixative were also used to fix peripheral olfactory organs that had not been cardiac perfused. The peripheral olfactory organ was then grossly dissected out of the animal under a Nikon SMZ-2B dissecting scope with NKII

Fiber Optic Light (Nikon, Canada, Mississsauga, ON) and stored in Zamboni's fixative until cryosectioning.

Before preparing round goby peripheral olfactory organs for transmission electron microscopy, fish were anaesthetized with MS-222, weighed and measured. Cardiac perfusion was performed using saline (6.3%) followed by Karnovsky's fixative. The nostrils were then removed and stored overnight in Karnovsky's fixative.

Cryosections

To prevent against the formation of ice crystals, Zamboni or paraformaldehyde-fixed adult round goby peripheral olfactory organs were cryoprotected through an ascending sucrose series: 10% sucrose for 1 hour, 20% sucrose for 1 hour, and 30% sucrose in distilled water overnight (Appendix A), at 4°C. Following sucrose infiltration, the tissue was placed in M-1 cryo-embedding matrix (1310, Thermo Shandon, Fairlawn, NJ) and placed in a vacuum chamber for 15 minutes. Afterwards they were frozen at -40°C in a Microm HM-500 OM cryostat (Zeiss; Heidelberg, Germany). Twenty micron thick sections were made and collected on Superfrost Plus slides (12-550-15, Fisher Chemicals, Fairlawn, NJ) (Appendix A) to ensure adhesion of the sections to the slide, allowed to air dry for at least an hour at room temperature then stored at -20°C overnight or until used. Prior to immunocytochemistry, slides were warmed to room temperature for 10 minutes, rehydrated for 10 minutes with 0.1 M phosphate buffered saline (PBS) (pH =7.4), cold fixed in acetone for 10 minutes, and rehydrated with 0.1 M PBS (pH = 7.4).

Immunocytochemistry (ICC):

Immunocytochemistry was performed on 20 micron sections as described by Zaidi *et al.* (1998) using primary antibodies acetylated tubulin (anti-mouse acetylated tubulin 1:1000, Sigma, St. Louis, MO, USA), and $G_{\alpha o}$ and $G_{\alpha olf}$ (anti-rabbit; diluted 1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (see Appendix A for procedure). Alexa (conjugated anti-rabbit and anti-mouse IgG; diluted 1:100; Molecular Probes, Eugene, Oregon, USA) was used as the secondary antibody and the sections were mounted with Vectashield® (Vector Labs, Burlingame, CA). Fluorescence microscopy and image analysis was performed on a Zeiss Axioskop FS (post-acquisition software: Northern Eclipse) and BioRad MRC 1024 Confocal Laser Scanning Microscope equipped with a Krypton/Argon laser.

Whole mounts were performed by removing the ventral surface of the nasal cavity from a fixed animal and applying the ICC procedure on this dissected tissue.

Changes in tissue processing for DAB immunocytochemistry included the use of the ABC Elite Kit (rabbit IgG for G protein antibodies) and DAB Fast tablets with metal enhancers (PK6101, Vector Labs, Burlingame, CA). These sections were dehydrated, mounted in Permount® (SP15-500, Fisher Chemicals, Fairlawn, NJ), then viewed with a Zeiss Axioskop FS.

Spatial Analysis of $G_{\alpha olf}$ and $G_{\alpha o}$ Immunoreactivity:

The distribution of olfactory epithelium in the olfactory chamber was assessed in sixteen male gobies (total length = 98.3 mm ± 4.28 and weight = 11.0 g ± 1.56) that were

serially sectioned in the vertical plane and prepared for G_{olf} and G_{o} immunocytochemistry. Each section was photographed and arranged serially for analysis.

Semi and ultrathin plastic sections for TEM:

Karnovsky-fixed tissue was osmicated in 1% osmium tetroxide (OsO_4), dehydrated through an ascending series of ice-cold ethanol, passed through propylene oxide and embedded into epoxy resin (see Appendix A for procedure). Semi-thin sections (1 μm) were taken on a RMC 6000 XL ultramicrotome (EM Lab Equipment Inc., Whitby, ON), stained with 1% toludine blue and viewed under a brightfield microscope until olfactory epithelium was seen in the sections.

For transmission electron microscopy, ultrathin sections (90 nm) were prepared with an ultramicrotome, collected onto copper electron microscope grids, stained with 7% uranyl acetate, and photographed on a Philips 201 transmission electron microscope to view ciliated and microvillar OSNs.

Film processing and scanning:

All TEM pictures were captured using Kodak® film and a 35 mm camera attached to the transmission electron microscope, the film was rewound and processed in the dark room with a red light. Kodak® T developer was used to develop the negatives on the 35 mm film. The film was fixed with Kodafix®, rinsed, and subsequently allowed to dry. The TEM images were magnified and Kodak® paper (Polycontast III RC paper) was exposed to the images. The Kodak® paper was placed in developer for 5 minutes and

then placed in a fixer for Hyperfilm. The photos were dried and scanned into the computer and the images edited (Adobe Photoshop Deluxe®).

RESULTS

Gross structure of the peripheral olfactory organ:

The head of the round goby contains a pair of nostrils each containing two nares, the tentacular anterior naris and the posterior naris (Figure 3 A). Impression casts of the nasal cavity show that the accessory nasal sacs are located adjacent and ventral to the posterior nostril (Figure 3 B). The nasal cavity was narrow at the tubular anterior naris and wider towards the posterior, with two bulbous shaped accessory nasal sacs adjacent to the posterior nostril (Figure 3 C, D). The ethmoidal sac was positioned at the medial axis and the lachrymal sac was located laterally (Figure 3 D). Both had a bulbous appearance and were connected by duct-like passages to the anterior olfactory chamber.

Acetylated tubulin immunocytochemistry of the nasal cavity showed the location of cilia and neuronal cell processes within the nasal cavity. Ciliated, acetylated tubulin immunoreactive surfaces lined the entire dorsal and ventral surfaces of the nasal cavity from the tentacular anterior nostril to the posterior nostril (Figure 4). The accessory nasal sacs were identified extending from the posterior ventral surface of the nasal cavity and were deficient of acetylated tubulin immunoreactivity (Figure 4 D). Epithelium with numerous acetylated tubulin immunoreactive nerve fascicles in the underlying lamina

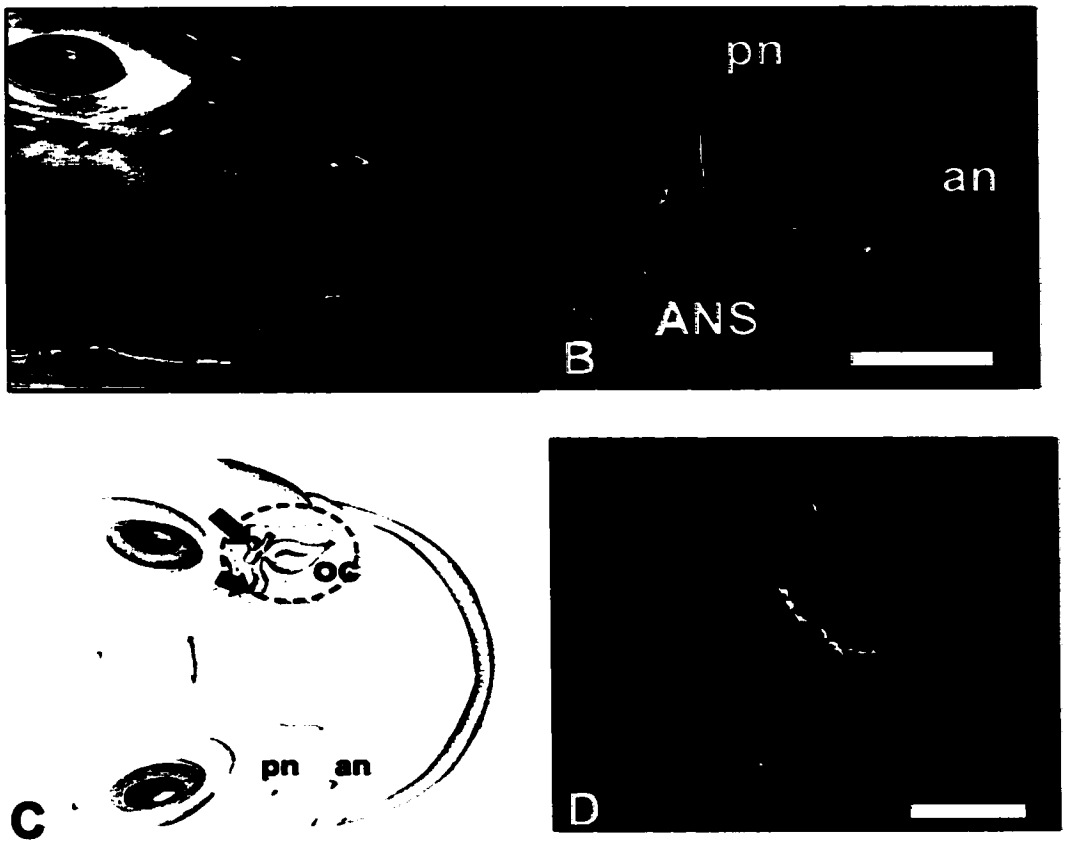

Figure 3: The nasal cavity of the round goby. (A) A photograph of the round goby head highlighting the placement of the tentacular anterior nostril (an) and the posterior nostril (pn). (B) A lateral view of the nasal mold demonstrates the posterior placement of the accessory nasal sacs (ANS) in relation to the anterior (an) and posterior (pn) nares. Scale bar is 1 mm. (C) A diagram showing the location of the nostrils and the nasal cavity. The location of the anterior nostril (an) and posterior nostril (pn) are shown. The area outlined by a dashed line in the upper portion of the diagram shows the three compartments of the nasal cavity, the olfactory chamber (OC), and two posterior accessory nasal sacs (arrows). Drawing by Cortney Smith. (D) A ventral view of the mold impression of the nasal cavity shows the olfactory chamber (OC) and two accessory nasal sacs. The ethmoidal sac (ES) is located medially, and the lachrymal sac (LS) is positioned laterally. Scale bar is 1 mm.

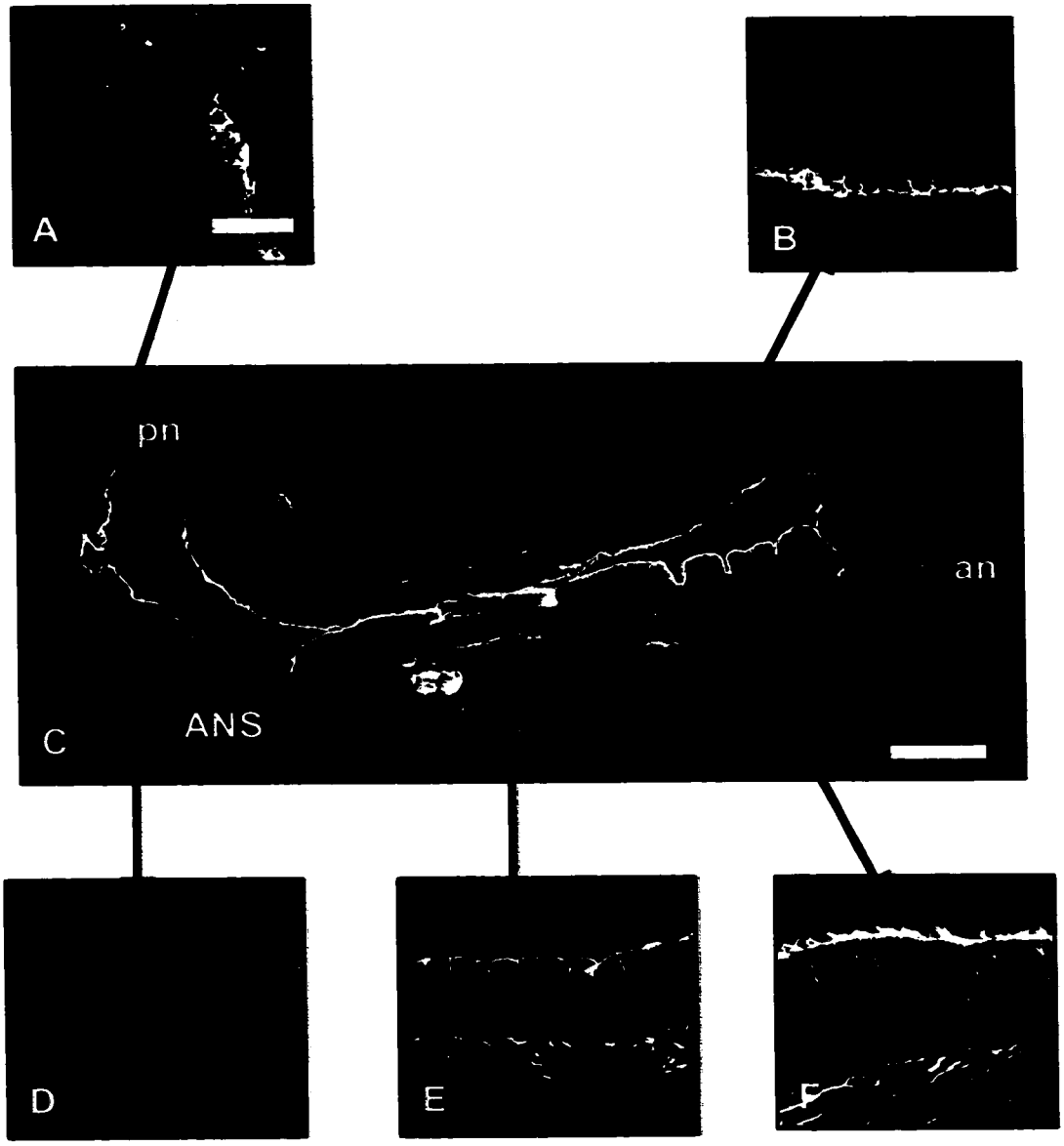

Figure 4: A longitudinal section of the nasal cavity (C) shows intense acetylated tubulin immunoreactivity extending along the dorsal epithelial surface (A and B) from the anterior (an) to the posterior nostril (pn). Surveys show that the immunoreactivity is prominent on the ventral surface (E and F) and absent from the accessory nasal sac (ANS) (D). The posterior nostril was lined by an acetylated tubulin immunoreactive on its apical surface. Scale bar for (C) is 100 μm and the scale bar for A, B, D, E, and F is 50 μm.

propria was recognized as olfactory epithelium (Figure 4 E, F and Figure 7 B) and was located on the ventral surface (Figure 4 C, D) in this sagittal longitudinal section of the peripheral olfactory organ (Figure 4 A, B). Two small depressions were seen in the anterior region of the olfactory epithelium (Figure 4 C). The epithelium lining the accessory nasal sacs was unciliated, and ciliated epithelium covered the surface of the nasal cavity adjacent to the posterior nostril (Figure 4 A, C, D).

Fine structure and distribution of olfactory sensory neurons:

Tissue resembling olfactory epithelium, with acetylated tubulin immunoreactive dendrites, axons, and nerve fascicles in the lamina propria, was seen in the longitudinal section (Figure 4 C, E, F). Cross sections revealed a single low lamella located along the medial axis low of the floor of the nasal cavity shown in serially sectioned preparations (Figure 5). The presence of acetylated tubulin-immunoreactive fibers extending into the lamina propria from the olfactory epithelium on the ventral, lateral and dorsal surfaces suggested that the spatial pattern for OSNs extended beyond the low olfactory lamella. The olfactory mucosa was clearly distinguished from non-olfactory surfaces by the presence of numerous acetylated tubulin-immunoreactive nonmyelinated nerve fascicles in the lamina propria. In the medial margin, fascicles converged into a prominent olfactory nerve (Figure 5).

Transmission electron microscopy confirmed the presence of ciliated and microvillar OSNs in the olfactory mucosa (Figure 6 A, B). Cilia extended from the olfactory knob, a small dome-like swelling, of ciliated OSNs (Figure 6 A). Microtubules

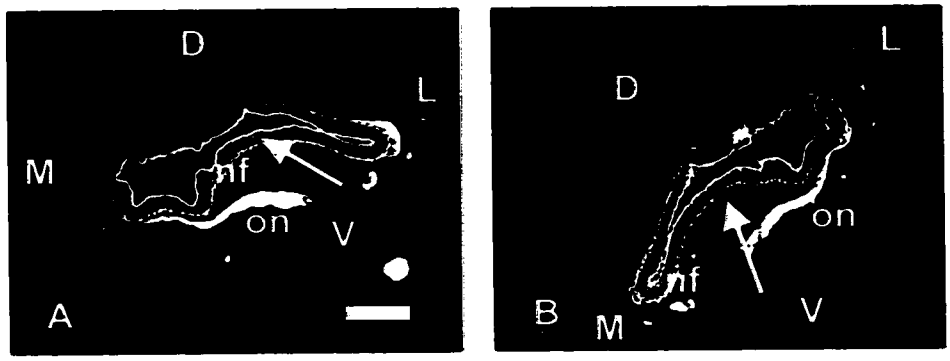

Figure 5: Cross sections through the peripheral olfactory organ. These cross sectional micrographs (A and B) show the nasal cavity with acetylated tubulin immunoreactivity in the olfactory muscosa; nerve fasicles (nf) are visible beneath the epithelium, on the medial (M), lateral (L), ventral (V), and dorsal (D) surfaces; nerve fasicles converge medially, forming a prominent olfactory nerve (on). Arrows denote a single low longitudinal lamella. Scale bar, shown in A is 200 μm.

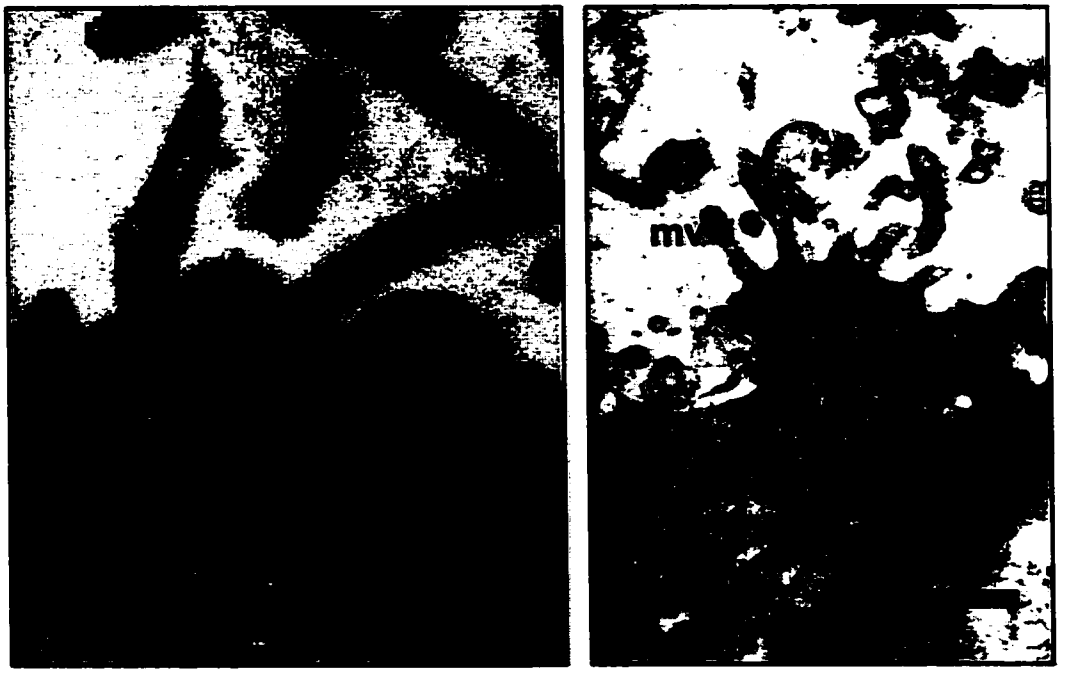

Figure 6: Transmission electron micrograph of a ciliated olfactory receptor neuron; two cilia (ci) protrude from the prominent olfactory knob; microtubules are abundant in the dendritic cytoplasm (arrows). Scale bar is 0.2 μm. (B) A tuft of microvilli (mv) extend from a low olfactory knob. Two centrioles (ce) are visible in the apical dendrite. Scale bar is 1 μm.

were abundant in the cytoplasm below the olfactory knob. The olfactory knob was less prominent in microvillar OSNs and short microvillar projections extended into the lumen (Figure 6 B). Centrioles were visible in the cytoplasm subjacent to the olfactory knob. Microvillar and ciliated OSNs were identified through transmission electron microscopy (Figure 6 A, B), and the distribution of these cells was assessed through immunocytochemistry against G proteins.

The $G_{\alpha olf}$ immunoreactive ciliated OSNs were evenly distributed in whole mount preparations (Figure 7 A). The cilia of ciliated OSNs were $G_{\alpha olf}$ immunoreactive (Figure 7 C), as were the dendrites, cell bodies and axons (Figure 7 D). The heavy ciliation on OSNs and nonsensory cells (Figure 7 B) prevented the viewing of the lower $G_{\alpha 0}$ immunoreactive microvilli in whole mount preparations, as microvilli (1 μm) are considerably shorter than cilia (10 μm). However, in sectioned tissue, the $G_{\alpha 0}$ immunoreactive microvilli, dendrites, cell bodies and axons were prominent (Figure 7 C). The nuclei of the $G_{\alpha 0}$ –immunoreactive OSNs were positioned within the upper third of the olfactory epithelium, in comparison to the more basal perikarya of $G_{\alpha olf}$ –immunoreactive OSNs (Figure 7 C, D). This perikaryal distribution implies that the $G_{\alpha 0}$ –immunoreactive OSNs are microvillar and the $G_{\alpha olf}$ –immunoreactive OSNs are ciliated (Morita and Finger 1998). In the round goby, the ciliated OSNs are $G_{\alpha olf}$ –immunoreactive, and the microvillar OSNs are $G_{\alpha 0}$ –immunoreactive .

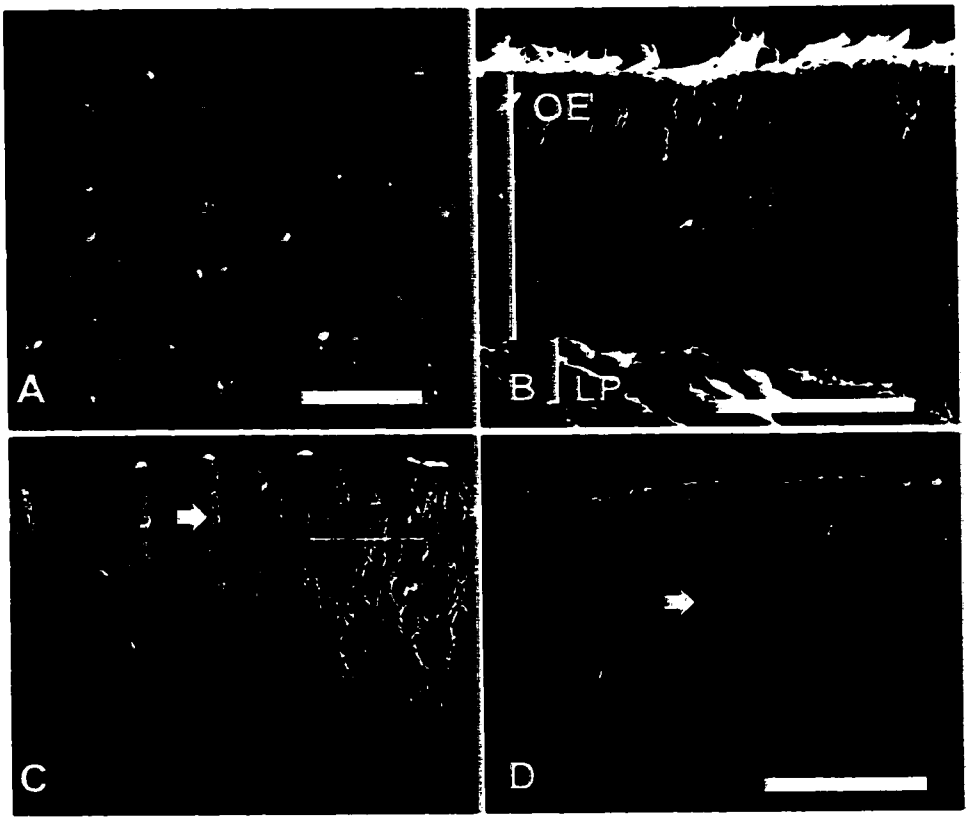

Figure 7: High power confocal images of preparations stained for $G_{\alpha olf}$ and $G_{\alpha o}$ immunofluorescence. (A) A whole mount view of the ventral luminal surface of the anterior olfactory chamber shows $G_{\alpha olf}$ – immunoreactive cilia. Scale bar is 10 μm. (B) A section stained for acetylated tubulin immunoreactivity shows the densely ciliated covering of the olfactory epithelium. The acetylated tubulin immunoreactivity is also seen in the OSN dendrites and axons within the olfactory epithelium (OE), and extends to axon fascicles within the lamina propria (LP). Scale bar is 50 μm. (C) Sectioned olfactory epithelium stained for $G_{\alpha o}$ immunocytochemistry shows the superficial location of the cell bodies of the immunoreactive cells, as well as the staining in the apical microvillar tuft, dendrite, cell body, and axon of presumptive microvillar OSNs. The arrow points to the dendrite of a single $G_{\alpha o}$ immunoreactive OSN. The scale bar is shown in D. (D) Sectioned olfactory epithelium stained for $G_{\alpha olf}$ immunocytochemistry shows labeling in the cilia, dendrites, cell bodies, and axons of OSNs. The arrow points to the dendrite of a $G_{\alpha olf}$ immunoreactive OSN. The perikarya are in the lower portion of the olfactory epithelium. Scale bar is 25 μm.

Spatial analysis of serially sectioned cross-sectional views of the olfactory chamber revealed the distribution of ciliated $G_{\alpha olf}$ immunoreactive OSNs and microvillar $G_{\alpha o}$ immunoreactive OSNs (representative sections shown in Figure 8). A low ventral lamellar ridge was evident from the anterior to the posterior edges of the olfactory chamber. Adjacent to the anterior nostril, olfactory epithelium that contained both $G_{\alpha olf}$ and $G_{\alpha o}$ immunoreactivity lined the ventral surface and the lateral edges (Figure 8 A, B). The $G_{\alpha olf}$ and $G_{\alpha o}$ immunoreactivity extended to the lateral and dorsal surfaces (Figure 8 C, D) in over 90% of the serial sections. At the posterior edge of the olfactory chamber, the olfactory epithelium was limited to the ventral olfactory lamella (Figure 8 E, F). High power views of the dorsal olfactory epithelium confirmed the presence of $G_{\alpha olf}$ and $G_{\alpha o}$ immunoreactivity on the OSN apical olfactory knobs and dendritic regions (Figure 9). These results show that olfactory epithelium with ciliated and microvillar OSNs lines the entire olfactory chamber from the anterior nostril to the accessory sacs, with the exception of small regions adjacent to the anterior and posterior nostril.

Cells that fit the description of crypt cells:

Cells that fit the description of crypt cells, bulbous in shape, with a projection reaching to the apical surface of the olfactory epithelium (Hansen and Finger 2000), were present in serial sections of olfactory epithelium of some specimens stained with $G_{\alpha o}$ immunocytochemistry (Figure 10 A). When viewed under high power, these cells showed intense $G_{\alpha o}$ immunoreactivity, with a prominent apical cilium that was acetylated tubulin-immunoreactive (Figure 10 B).

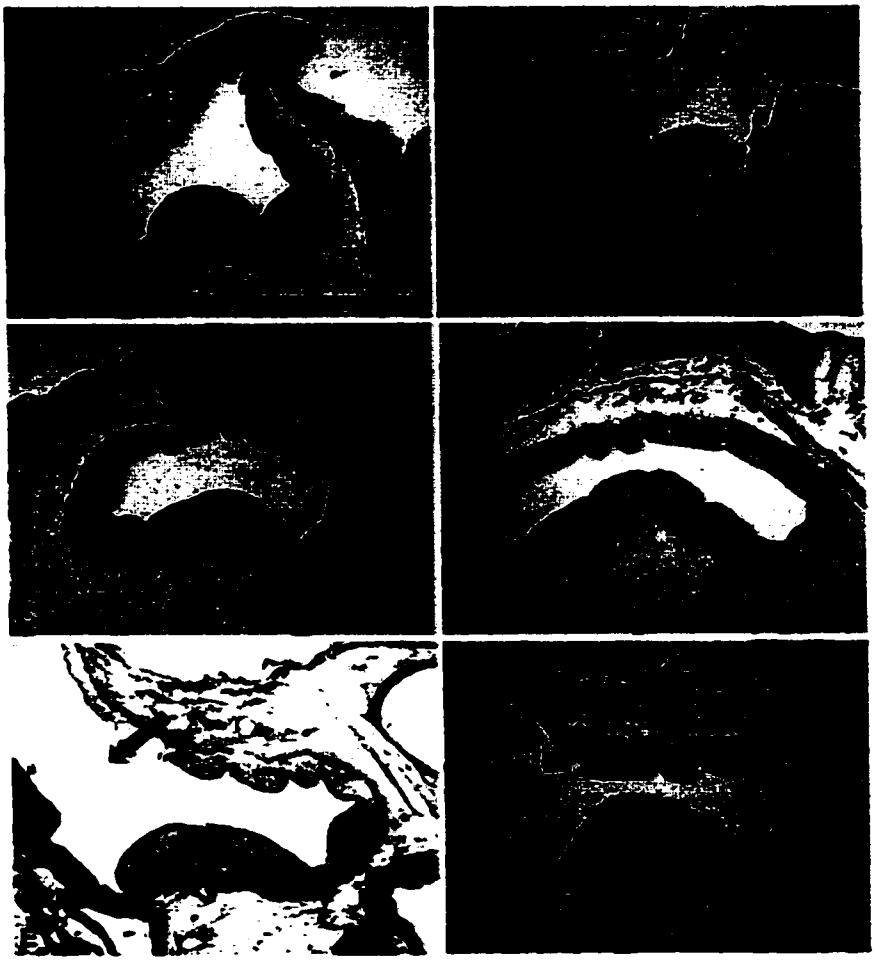

Figure 8: Low power cross-sectional views of the olfactory mucosa in the Round goby olfactory chamber stained for $G_{\alpha olf}$ and $G_{\alpha o}$ immunoreactivity. All Micrographs have the same magnification. Scale bar, shown in A, is 200 μm. A, C, and E show $G_{\alpha olf}$ immunoreactivity. B, D, and F are $G_{\alpha o}$ immunoreactive. A and B show $G_{\alpha olf}$ and $G_{\alpha o}$ immunoreactivity on the ventral and lateral surfaces at the anterior edge of the olfactory chamber, adjacent to the anterior nasal cavity. The arrow points to epidermal tissue covering the anterior nostril. B and D show $G_{\alpha olf}$ and $G_{\alpha o}$ immunoreactivity in representative sections along the length of the olfactory chamber. $G_{\alpha olf}$ and $G_{\alpha o}$ immunoreactivity is is located on the ventral Olfactory lamella, as well as the lateral and dorsal surfaces. The medial dorsal edge has scalloped outline and lacks $G_{\alpha olf}$ immunoreactivity in the lamina propria (arrow). E and F show $G_{\alpha olf}$ and $G_{\alpha o}$ immunoreactivity at the caudal edge of the olfactory chamber, where staining is limited to the ventral olfactory lamella. The arrow points to the posterior nares. Nerve fasicles are seen converging ventral to the Lamella (nf). Preparation by Cortney Smith.

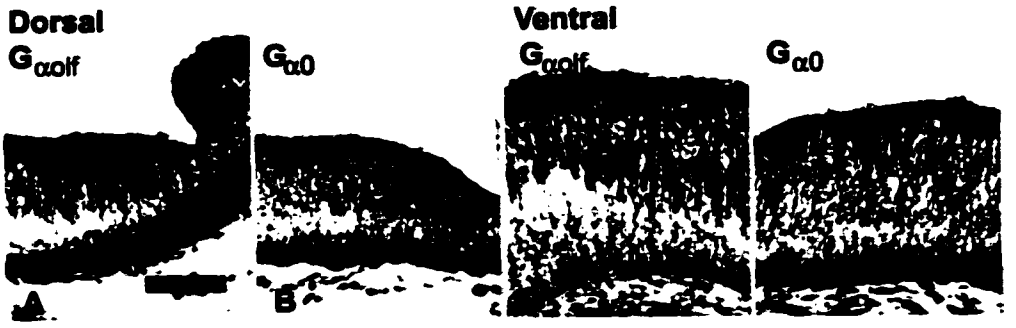

Figure 9: High power views of $G_{\alpha olf}$ and $G_{\alpha 0}$ immunoreactivity on the dorsal and ventral surface of the olfactory chamber. Scale bar shown in A is 50 μm. A and C show $G_{\alpha olf}$ immunoreactivity; B and D show $G_{\alpha 0}$ immunoreactivity. A and B show $G_{\alpha olf}$ and $G_{\alpha 0}$ immunoreactivity on the dorsal surface of the olfactory chamber. The $G_{\alpha olf}$ immunoreactivity extends deep into the olfactory epithelium (A) and the $G_{\alpha 0}$ immunoreactivity (B) is limited to the upper third of the olfactory epithelium. C and D show the ventral surface of the olfactory chamber. The olfactory epithelium on the ventral surface is thicker than on the dorsal surface. $G_{\alpha olf}$ immunoreactivity reaches to the lower third of the olfactory epithelium (C), and $G_{\alpha 0}$ immunoreactivity remains in the upper third of the olfactory epithelium.

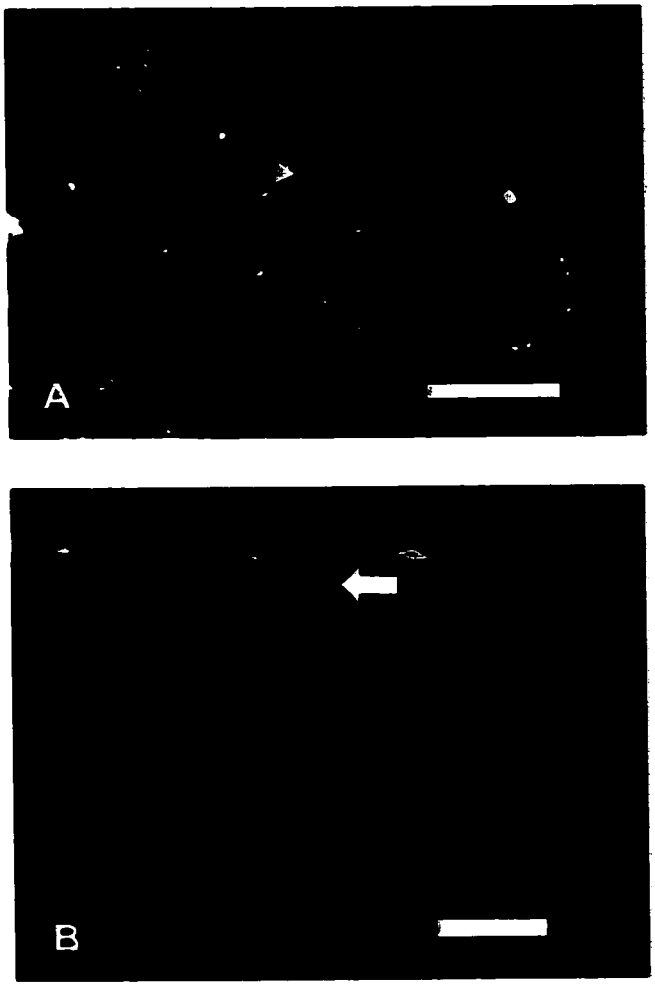

Figure 10: Micrographs showing $G_{\alpha o}$ immunoreactive crypt cells. (A) A low powered section through the peripheral olfactory organ of the round goby shows $G_{\alpha o}$ immunoreactive crypt cells distributed randomly. Scale bar is 50 μm (B) A crypt cell double labeled with $G_{\alpha o}$ immunocytochemistry (green), and acetylated tubulin immunocytochemistry (red). The crypt cell is strongly $G_{\alpha o}$ immunoreactive, with an acetylated tubulin immunoreactive distal tip (arrow). Scale bar is 20 μm.

DISCUSSION

This first comprehensive study of the peripheral olfactory organ in a perciform teleost demonstrates morphological adaptations for maximizing the use of olfactory sensory cues. In the round goby, these include prominent accessory nasal sacs and a tube-shaped unilamellar olfactory chamber with microvillar and ciliated OSNs covering the ventral, lateral and dorsal surfaces of the olfactory chamber.

Gross structure:

In teleost fish, water enters the nose through the anterior nares and exits through the posterior nares. This may happen passively through locomotion of the fish in water or actively by ciliary action within the pits. Intense acetylated tubulin immunoreactivity along the epithelial surface of the entire nasal cavity indicates that water flows through the nasal cavity of the round goby with the assistance of ciliary action. Passive flow of water through the nostril is not likely in the round goby because the anterior nostril opening is narrow, with a narrow tubular opening. This skin covering the anterior nares may have evolved to keep benthic debris out of the nose.

The round goby contained two accessory nasal sacs, the lateral lachrymal sac and the medial ethmoidal sac. This is a novel finding, as previous research on gobiids has not identified this structure. A tendon or other connective tissue may attach the accessory nasal sac to the gills, so that gill movements control expansion and contraction of the sac (Melinkat and Zeiske 1979). The occurrence of one or two accessory nasal sacs in teleost

fish (summarized in Table 1) appears to follow a phylogenetic pattern, with two accessory sacs present in the peripheral olfactory organ of fish in the order Perciformes. Three exceptions are *Trichinus vipera*, *Sphyraena cameroonii* and *Scomber scombrus*, with a single accessory nasal sac (Burne 1909). Fish in the order Pleuronectiformes contained a fused pair (Kyle 1899; Burne 1909; Chabanaud 1927; Applebaum and Schemmel 1983; Lierman 1933; Webb 1993) or single accessory nasal sacs (Webb 1993). The accessory nasal sacs in the round goby were round in shape, and comprised half the length of the olfactory chamber, as were those of another perciform *Thynnus thunnia* (Sinha and Sinha 1990) and the Pleuronecidae (Webb 1993).

The presence of these prominent accessory sacs in the round goby implies that it has the capacity to regulate the flow of water over the surface of the OSNs. A pumping mechanism regulating intake and expulsion of water through the olfactory organ of fish with accessory nasal sacs has been previously demonstrated in other perciforms (e.g., Burne 1909; Døving *et al.* 1977; Sinha and Sinha 1990). This ventilatory mechanism is based upon constant compression and decompression of the sacs by contraction of surrounding buccal muscles and the movement of maxillary bones or pressure changes in the lymphatic system (van den Berghe 1929; Melinkat and Zeiske 1979). The occurrence of two accessory nasal sacs in the round goby designates it a cyclomate. The heavy ciliation of olfactory and non-sensory epithelium allows the flow of water through the nasal cavity to be regulated by both ciliary beating and accessory sac compression (Døving *et al.* 1977). The prominent accessory nasal sacs and dense ciliation of the nasal cavity in the round goby suggest that odourant molecules reach the OSNs through

Table 1: A summary of the number of nasal sacs in teleost fishes (subdivision Eutelaostei). The taxonomic classification scheme follows Nelson (1994). Genus and species names are listed alphabetically within family.

Order	Family	Genus species	Number of Nasal Sacs	Reference
Osteoglossiformes	Mormyridae	*Mormyrus sp.*	1	Burne 1909
	Gymnarchidae	*Gymnarchus niloticus*	0	Burne 1909
Anguilliformes	Anguillidae	*Anguilla vulgaris*	0	Burne 1909
	Myrocongridae	*Muraena tigrina*	0	Burne 1909
	Congridae	*Conger vulgaris*	0	Burne 1909
Clupeiformes	Chirocentridae	*Chirocentrus dorab*	0	Burne 1909
	Clupeidae	*Clupea harengus*	0	Burne 1909
Cypriniformes	Cyprinidae	*Cyprinius carpio*	0	Doving *et al.* 1977
		Labeo rohita	1	Kapoor & Ojha 1972
		Rutilis rutilis	0	Doving *et al.* 1977
		Tinca tinca	0	Doving *et al.* 1977
		Tinca vulgaris	0	Burne 1909
Siluriformes	Ictaluridae	*Ictalurus sp.*	0	Atema, *pers. comm.*
	Siluridae	*Siluris glanis*	0	Burne 1909; Doving *et al.* 1977
	Clariidae	*Clarias lazera*	1	Burne 1909
	Malapteruridae	*Malapterurus electricus*	1	Burne 1909
	Pimelodidae	*Pimelodus seboe*	0	Burne 1909
Gymnotiformes	Gymnotidae	*Gymnotus electricus*	0	Burne 1909
Esociformes	Esocidae	*Esox lucius*	0	Burne 1909
Osmeriformes	Osmeridae	*Osmerus eperlanus*	1	Burne 1909
Salmoniformes	Salmonidae	*Coregonus oxyrhynchus*	1	Burne 1909
		Salmo solar	1	Burne 1909
Myctophiformes	Myctophidae	*Myctophum punctatum*	0	Burne 1909
Gadiformes	Phycidae	*Gaidropsarus vulgaris*	1	Burne 1909
	Merlucciidae	*Merluccius vulgaris*	1	Burne 1909
	Gadidae	*Gadus sp.*	0	Burne 1909
Lophiiformes	Lophiidae	*Lophius piscatorius*	0	Burne 1909
Mugiliformes	Mugilidae	*Mugil chelo*	2	Burne 1909
Atheriniformes	Bedottidae	*Bedotia geayi*	1	Melinkat & Zeiske 1979
	Melanotaeniidae	*Chilatherina sentaniensis*	1	Zeiske *et al.* 1979
		Glossolepis incisus	1	Zeiske *et al.* 1979
		Melanotaena fluviatilis	1	Zeiske *et al.* 1979
		Melanotaena maccullochi	1	Zeiske *et al.* 1979
		Melanotaena maculata	1	Zeiske *et al.* 1979
	Atherinidae	*Craterocephalus sp.*	1	Zeiske *et al.* 1979

Table 1 continued:

Beloniformes	Belonidae	*Belone belone*	0	Burne 1909
	Exocoetidae	*Exocoetus volitans*	0	Burne 1909
	Hemiramphidae	*Hemiramphidae sp.*	0	Burne 1909
Cyprinodontiformes	Anablepidae	*Anableps microlepis*	1	Burne 1909
	Poeciliidae	*Xiphophorus helleri*	1	Kux *et al.* 1988
	Cypinodontidae	*Orestias lesueuri*	1	Burne 1909
Beryciformes	Berycidae	*Beryx delphinus*	2	Burne 1909
Zeiformes	Zeidae	*Zeus faber*	1	Burne 1909
	Caproidae	*Capros aper*	2	Burne 1909
Gasterosteiformes	Gasterosteidae	*Gasterosteus spp.*	1	Burne 1909
		Spinachia spinachia	1	Theisen 1982
	Fistulariidae	*Fistularia spp.*	1	Burne 1909
		Sebastes melanops	2	Johnson & Brown 1962
Scorpaeniformes	Scorpaenidae	*Scorpaena porcus*	present*	Doving *et al.* 1977
	Triglidae	*Trigla hirundo*	2	Burne 1909
	Cyclopteridae	*Cyclopterus lumpus*	2	Burne 1909
	Centrarchidae	*Centrarchid sp.*	2	Eaton 1956
Perciformes	Percidae	*Perca fluviatilis*	2	Burne 1909; Doving *et al.* 1977
	Sparidae	*Pagellus centronotus*	2	Burne 1909
	Mullidae	*Mullus barbatus*	2	Burne 1909
	Latrididae	*Latridopsis ciliaris*	2	Burne 1909
	Cichlidae	*Tilapia sp.*	present*	Doving *et al.* 1977
	Labridae	*Crenilabrus cinereus*	present*	Doving *et al.* 1977
		Labrus bergylta	present*	Doving *et al.* 1977
	Bovichthyidae	*Bovichthys variegatus*	2	Burne 1909
	Trachinidae	*Trachinus vipera*	1	Burne 1909
	Gobiidae	***N. melanostomus***	**2**	**This study**
	Schyraenidae	*Sphyraena cameroonii*	1	Burne 1909
	Scombridae	*Scomber scombrus*	1	Burne 1909
		Thunnus thunnina	2	Sinha & Sinha 1990
	Channidae	*Channa punctatus*	2	Kapoor & Ojha 1972; 1973
		Channa marulia	0	Burne 1909
Pleuronectiformes	Scophthalmidae	*Scophthalmus sp.*	2	Kyle 1899
	Pleuronectidae	*Ammotretis rostratus*	1	Webb 1993
		Pleuronectes sp.	2	Burne 1909; Liermann 1933; Webb 1993
	Soleidae	*Solea vulgaris*	Fused pair	Burne 1909; Chabanaud 1927; Applebaum & Schemmel 1983
	Cynoglossidae	*Cynoglossus sp.*	Fused pair	Kyle 1899; Webb 1993
		Symphurus atricauda	Fused pair	Webb 1993

* This paper shows the presence of accessory nasal sacs but does not indicate the number of sacs.

"sniffing" (Nevitt 1991) as well as ciliary beating. As round gobies perch on substrates using their fused pelvic fin (personal observation), the ability to sample surrounding waters for olfactory cues through a pumping mechanism would provide an evolutionary advantage to these small fish. Furthermore, previous studies that linked round goby gill ventilatory responses to olfactory stimulation by putative pheromones of steroidal origin have suggested that the gill ventilatory mechanism is associated with increasing water flow through the olfactory organ (Murphy *et al.* 2001; Murphy and Stacey 2002).

Fine structure:

The peripheral olfactory organ of the round goby contains a relatively flat surface of olfactory epithelium with one low lamella along the rostro-caudal axis. This structure varies from that seen in most teleost fishes. Usually the peripheral olfactory organ is a multi-lamellar (folded) structure (Yamamoto 1982). Olfactory ability is not directly related to the number of lamellae present in the nasal cavity and there is no simple relationship between the number of lamellae and the acuity of olfactory sense (Pipping 1926, 1927). Using gross dissection and scanning electron microscopy of the peripheral olfactory organ, Yamamoto (1982) showed that other gobiids (e.g., *Acanthogobius flavimanus*, *Odontamblyopus rubicundus*) in the order Perciformes contain a single, centrally located olfactory lamellae. Yamamoto (1982) also listed other species containing one olfactory lamella (e.g., *Ammodytes personatus*, *Navodon modestus*) and confirmed that olfactory ability is not directly related to the surface area in the olfactory organ. Fish with a similar demersal or reef associated lifestyle are typically found to contain 0 or 1 (longitudinal) olfactory lamellae (summarized in Table 2), whereas pelagic fish typically have 1 (transverse) to several lamellae (e.g., *Thunnus thynnus*, *Leiognathus*

Table 2: A summary of fish lifestyle in relation to the number of olfactory lamellae as classified by Yamamoto (1982).

Family	Fish species	Number of Lamellae	Lifestyle
Gobiesocidae	*Conidens laticephalus*	0	Demersal*
Adrianichthyidae	*Oryzias latipes*	0	Benthopelagic**
Poeciliidae	*Gambusia affinis*	0	Benthopelagic
Syngnathidae	*Hippocampus coonatus*	0	Demersal
Synbranchidae	*Fluta alba*	0	Demersal
Blennidae	*Omobranchus elegans*	0	Demersal
Monacanthidae	*Rudarius arcodes*	0	Reef-Associated***
Ostraciidae	*Ostracion tuberculatus*	0	Reef-Associated
Gobiidae	***Neogobius melanostomus****	**1**	**Demersal**
Gobiidae	*Acanthogobius flavimanus*	1	Demersal
Gobiidae	*Odontamblyopus rubicundus*	1	Demersal
Ammodytidae	*Ammodytes personaltus*	1	Demersal
Monacanthidae	*Navodon modestus*	1	Demersal
Hemiramphidae	*Hemiramphus sajori*	1*	Pelagic****
Exocoetidae	*Cheilopogon agoo*	1*	Pelagic
Scomberesocidae	*Cololabis saira*	1*	Pelagic
Leiognathidae	*Leiognathus nuchalis*	29	Pelagic
Toxotidae	*Toxotes jaculator*	12	Pelagic
Sphyraenidae	*Sphyraena japonica*	26	Pelagic
Scombridae	*Scomber japonicus*	25	Pelagic
Scombridae	*Thunnus thynnus*	40	Pelagic

*transverse lamellae, as opposed to a longitudinal lamellae seen in other fishes with a single lamellae.

** refers to the species used in this study

*living on or near the sea or lake bottom

**associated equally with the water and sea or lake bottom

***always associated with or spending much time on a reef

****swimming actively, rather than living on the sea or lake bottom

nuchalis. Toxtes jaculator. Hemiramphus sajori. Cheilopogon agoo. Cololabis saira). demersal fish with a single longitudinal lamella may be more hydrodynamic than those with a single transverse lamella or several lamellae. Water would be moving with minimal resistance through the peripheral olfactory organ. Table 2 demonstrates that the olfactory chamber of some demersal fish may contain several lamellae, but the pattern is that all pelagic fish contain either 1 transverse lamellae or several lamellae.

In the round goby, olfactory epithelium lined the luminal surfaces of a unilamellar olfactory chamber with a rich supply of ciliated and microvillar OSNs, stretching from the anterior naris to the accessory nasal sacs. The rostral location of the OSNs, close to the anterior naris, may assist the goby with odor sampling while remaining stationary. There is a single previous report of olfactory epithelium in anterior and dorsal location in the bottom- dwelling sea stickleback, *Spinachia spinachia* (Gasterosteiformes, Gasterosteidae) (Theisen 1982). However in most teleosts, OSNs appear to be restricted to olfactory lamellae that line the floor of the nasal cavity (Yamamoto 1982). The presence of OSNs on the lateral and dorsal regions of olfactory chamber in the round goby likely maximizes the surface area for odourant/receptor interaction, as the goby possesses a unilamellar olfactory organ, compared with the multilamellar structures observed in many teleosts (Yamamoto 1982). Interestingly, in amphibians both dorsal and ventral surfaces of the relatively flat nasal cavity are covered by olfactory mucosa (Hansen *et al.* 1998).

G protein immunocytochemistry:

The use of G protein immunocytochemistry for $G_{\alpha olf}$ and $G_{\alpha o}$ was effective in assessing the distribution of ciliated and microvillar OSNs. The $G_{\alpha o}$ protein is expressed by vomeronasal sensory neurons in terrestrial vertebrates (Matsuoka *et al.* 2001), and is localized in microvillar OSNs in catfish and goldfish (Hansen *et al.* 2001). Both $G_{\alpha olf}$ and $G_{\alpha o}$ immunoreactive OSNs appeared to be equally prominent throughout the olfactory epithelium on the ventral, lateral and dorsal surfaces of the olfactory chamber. This even distribution pattern of ciliated and microvillar OSNs is common in teleosts. However other fish, such as Antheriniformes, show specific spatial mapping of ciliated and microvillar cell types (Zeiske *et al.* 1979; Yamamoto 1982).

Crypt cells, as described by Hansen and Finger (2000), were stained intensely with the $G_{\alpha o}$ antibody, suggesting that crypt cells may share similar antigenic properties to the alpha subunit of the G-protein for microvillar receptor neurons. As with other teleosts, round goby crypt cells varied in location and were absent in some specimens, while numerous in others (Hansen and Finger 2000). The function of crypt cells is unknown but it has been suggested that they may mediate responses to pheromones (Hansen and Finger 2000; Sorensen *et al.* 2002; Hansen *et al.* 2002).

Summary:

In summary, the peripheral olfactory organ in the round goby is like that of most Perciformes in that it contains two accessory nasal sacs, lachrymal and ethmoidal. Olfactory epithelium covers the dorsal, lateral and ventral surfaces of the unilamellar

olfactory chamber, with the exception of the rostral and caudal edges. Microvillar and ciliated OSNs are distributed throughout the olfactory epithelium, with G-protein immunocytochemistry serving to demonstrate this distribution. This widespread distribution of olfactory epithelium in the olfactory chamber is novel for the teleost peripheral olfactory organ, and is reminiscent of the amphibian peripheral olfactory organ. The spatial structure of the round goby olfactory system was discerned for the purpose of relating behavioural responses of the round goby to sex pheromones perceived during reproductive activities. Knowledge of the organization of the peripheral olfactory organ will allow for olfactory deprivation experiments and research showing cellular responses to sex pheromones released by conspecifics.

CHAPTER 2: Behavioural responses by the round goby, *Neogobius melanostomus* (Teleostei: Gobiidae), to putative pheromones and conspecific extracts.

INTRODUCTION

Functions of hormonal pheromones in fish:

Fish are constantly immersed in their physical and chemical environment, and their sensory systems are in continuous interactions with environmental changes (Hara 1993). Chemosensory systems of fish must be extremely well developed and serve to mediate many behaviours of fundamental importance (e.g. food-finding, predator avoidance, and intraspecific communication) (Colombo *et al.* 1982; Liley 1982; Sorensen 1992; Stacey *et al.* 1986; 1987; review by Sorensen and Caprio 1998). Understanding of the nature of odours recognized by the fish olfactory system is largely derived from behavioural and electrophysiological studies in conjunction with biochemical assays. There are four major structural classes of odourants described in several species of fish: (1) amino acids; (2) bile acids; (3) gonadal steroids and derivatives; and (4) prostaglandins (review by Hara 1993; review by Sorensen and Caprio 1998). Using highly specific olfactory receptor systems, extremely complex mixtures of these chemicals can be distinguished by fish, allowing them to represent various chemical messages (review by Sorensen and Caprio 1998). Murphy *et al.* (2001) tested these groups of odourants on round gobies by EOG and behaviour bioassays, and showed that round gobies responded to amino acids, bile acids, and steroids .

Many studies show the existence of chemical mediation in the reproductive behaviour of a variety of families of fish (e.g., Gobiidae (Tavolga 1956; Colombo *et al.* 1980), Cyprinindae (Lambert *et al.* 1986; Sorensen and Stacey 1990; Sorensen 1992); Cottidae (Dmitrieva and Ostroumov 1986; Dmitrieva *et al.* 1988); Cariidae (Lambert *et al.* 1986; Resink *et al.* 1987; 1989a; 1989b). It is likely that chemical signals do play an important role in reproduction by ensuring both physical and physiological synchronization of partners (Liley and Stacey 1983). The following behavioural interactions have been reported to be mediated by sex pheromones: (1) attraction of the male by the female, (2) attraction of the male by the ovulated or responsive female only, with stimulation of male courtship, (3) increase of male to male interactions induced by the female, (4) attraction of the female by the male, and (5) intrasexual attraction (Colombo *et al.* 1980).

All species of fish studied, when sexually mature, signal their stage of sexual maturity to conspecifics with pheromones and these pheromones have been found to detected by the olfactory system (Sorensen 1992; Stacey *et al.* 1986). These cues serve a wide variety of functions in different species and can be categorized as being either "primers", which evoke primarily endocrinological responses, or "releasers", which trigger behavioural changes (Bronmark and Miner 1992). Although pheromone production is generally associated with females, examples exist of nest guarding males which release cues to attract females (Colombo *et al.* 1980; Stacey *et al.* 1986). Sex steroids elicit physiological responses in fish at concentrations ranging from 10^{-4} to 10^{-13} M (e.g., *Ictalurus punctatus, Carassius auratus*) (Caprio 1978, Sorensen *et al.* 1987;

1988) and are species specific (Hara 1993). In goldfish post ovulatory pheromones are known to stimulate male sexual arousal and can be detected at 10^{-10}M in concentration (Sorensen *et al.* 1988).

Round gobies may use pheromones as a method of chemical communication. They live in benthic habitats where light levels are low and males are often sequestered in dark nests. Male round gobies actively and aggressively defend their nest sites fending off intruders and conspecifics of the same and opposite sex (Wickett and Corkum 1998; Corkum *et al.* 1998). In the absence of the visual cues, a gravid female, ready to oviposit, may give off a chemical signal so that the guarding males will allow her to enter the nest. It has been shown that other gobiids may use pheromones for communication during mating (Tavolga 1956; Colombo *et al.* 1980).

Mating strategies and hormonal pheromones in gobiids:

The gobies, global in distribution and comprising about 10% of extant teleost species (Miller 1984), are excellent subjects for sex pheromone research. The common gobiid reproductive pattern, in which territorial males defend a nest site which ovulated females must locate, presents a favourable situation for the evolution of male attractive pheromone(s). Pheromones are often important from recognition of sex and sexual condition in gobiids (Tavolga 1956; Colombo *et al.* 1982) and a relatively small set of studies provide a basis for pheromonal communication in gobies. The first study of a chemical communication system in a goby was done using the blind goby (*Typhlogobius californiensis*) (MacGinitie 1939). Chemoreception plays a role in monogamous pair

formation in the blind goby (MacGinitie 1939). The blind goby lives commensally in pairs in the burrows of the shrimp, *Callianassa affinis* and likely relies completely on chemical communication for its survival (MacGinitie 1939). These gobies are sensitive to invasion of their burrows by other gobies of their own sex. Aggressive behaviour is displayed among gobies of the same sex; passive behaviour is exhibited between gobies of the opposite sex (MacGinitie 1939). The recognition of the same sex invaders is entirely chemotropic, but the complete role of olfaction in this species is still unknown (MacGinitie 1939).

Sex discrimination and courtship behaviour through olfaction was discovered by Tavolga (1956) in *Bathygobius soporator*, a species within the same subfamily (Gobionellinae) as the round goby. He used a laboratory experiment to show that mature males "exploded into courtship" when exposed to gravid-female-water that originated from ovarian fluid secretions. The secretion was potent. Just 0.1 mL of the ovarian fluid in 5 gallons of water (1:10,000), evoked male courtship displays within 5-10 seconds after the introduction of water and displays lasted for up to 30 minutes. Anosmic males, whose nostrils were cauterized, did not respond to any amount of the courtship stimulating substance.

Colombo *et al.* (1977, 1980) showed that male gobies also appear to release reproductive pheromones. The leydig cell rich, mesorchial gland of the black goby contained conjugated reduced androgens, a major conjugate being etiocholanolone-glucuronide (ETIO-g; a 5β-reduced androgen conjugated to glucuronic acid). Other studies showed that this pheromone is a steroid hormone metabolite (Colombo *et al.*

1977, 1979,1980,1982). Colombo *et al.* (1979,1980) tested the behavioural actions of this compound and found that ovulated females were attracted to artificial nests associated with ETIO-g, whereas non-ovulated females were not. Behavioural studies also showed that gravid females approached and remained at source emitting this steroid source and were often induced to oviposit in the absence of males (Colombo *et al.* 1980).

Recently, Murphy *et al.* (2001) tested olfactory responses in the round goby to 8 prostaglandins and 114 steroids through use of behavioural responses and the electro-olfactogram (EOG), a physiological technique that displays extracellular voltage changes presumed to be generator potentials from OSNs (Murphy *et al.* 2001). Although prostaglandins failed to elicit EOG responses, 19 steroids met the criterion of inducing EOG responses of over 1 mV. Sexually dimorphic behavioural responses were seen when gill ventilation increase was used as a behavioural bioassay. The glucuronide form of ETIO-g and the non-glucuronide form of ETIO-g, ETIO, elicited both EOG responses (1 mV and 3mV respectively) and a relatively high increase in basal ventilation rate in both males and females. Estrone (E1) also produced ventilatory responses in males but did not produce any significant responses in females, suggesting that this pheromone may be produced by the female as a chemical stimulant. Murphy *et al.* (2001) suggested that round gobies from the Detroit River use a variety of chemical signals to attract females and that male round goby may also use ETIO-g/ ETIO to detect the reproductive status or proximity of neighbouring males, whereas females may use ETIO-g ETIO to search for mates.

These research studies form a solid basis for investigation into the identity of

reproductive pheromones released by the round goby females. Application of this knowledge has the potential of leading to the development of a pheromone trap for reproductive assessment and round goby population control in habitats where native species spawning sites are threatened by predation and competition by round gobies.

Olfactory sensory deprivation:

Copper sulfate treatments have been used repeatedly to diminish the occurrence of functional olfactory sensory neurons in teleost fish. Chronic copper sulfate exposure induces specific degeneration of olfactory sensory cells in rainbow trout (Julliard *et al.* 1993). Copper sulfate is known to specifically induce OSN death in fish olfactory epithelium (Julliard *et al.* 1996). Winberg *et al.* (1992) have shown that there is a significant decrease in EOG amplitude with increasing dissolved Cu^{2+} concentration. For these reasons researchers typically use copper to render fish anosmic for behavioural studies (e.g., Sorensen 2001). Effectiveness of neuronal cell death can be verified by using immunocytochemistry with antibodies against G proteins found on the membrane of OSNs.

Although copper sulfate has been used for olfactory sensory deprivation in behaviour bioassays and EOG recordings, it is also known to effect the behaviour of the fish. In a literature review, Atchison *et al.* (1987) found that copper has negative effects on fish behaviour including locomotion, attraction and avoidance, swimming performance, respiratory behavior, learning, social interactions, reproductive behavior, feeding, and predator avoidance. Altered behavior also may be a result of toxicant

damage to non-olfactory components of the nervous system (Atchison *et al.* 1987). At the morphological level, toxicants may damage nerve cell bodies, axons, and myelin sheaths (Weis *et al.* 2001). At the biochemical level, toxicants can alter the synthesis and release of neurotransmitters, which may be associated with behavioral changes (Weis *et al.* 2001).

To avoid negative non-olfactory effects of copper on fishes needed for research involving olfactory sensory deprivation, other forms of nasal occlusions are available including nasal plugging. By plugging the nasal passageway with an inert substance, the researcher is avoiding sublethal behavioural side effects observed when chemical agents are used for olfactory sensory deprivation. Classical research by Wisby and Hasler (1954) showed that plugging the nasal passages of salmon (*Oncorhynchus kisutch*) with cotton and petroleum jelly during their spawning migration was an effective olfactory deprivation technique as it affected their ability to locate their natal stream. Jahn (1969) also used petroleum jelly to occlude the nostrils and found that fewer anosmic cutthroat trout, *Oncorhynchus clarki*, individuals homed.

Olfaction-Ventilation Reflex Assay:

In teleost fish, there are considerable variations in size and structure of the peripheral olfactory organ. Typically, a current of water enters the anterior and leaves the posterior nostril either passively through the locomotion of the fish in water, actively by ciliary action within the peripheral olfactory organ, by the pumping action of the nasal sacs generally with the aid of one or more accessory nasal sacs, or a combination of all of

these (Hara 1975, Melinkat and Zeiske 1979). The peripheral olfactory organ in most teleosts contains either a pair of accessory nasal sacs or a single accessory nasal sac per nostril (Burne 1909; Belanger *et al. submitted*). The accessory nasal sacs are expanded and compressed when the mouth is opened and closed. Johnson and Brown (1962) state that the accessory nasal sacs in the black rockfish are attached by connective tissue to the premaxillary, palatine, and ethmoidal bones and to the under surface of the central chamber. The accessory nasal sacs are collapsed when the jaws are closed and stretches to full size when the jaws are opened.

Carmine was placed by Johnson and Brown (1962) at the openings of both anterior and posterior nostrils of partially anaesthetized black rockfish. When the mouth of the fish was opened and the gills expanded, considerable carmine was drawn into the anterior nostril but very little was drawn into the posterior nostril. When the mouth was closed and the gills contracted, carmine was expelled from the posterior nostril but none from the anterior. Thus accessory nasal sacs can be pumped to evoke "sniffing" (Nevitt 1991) and ventilation (opercular beats) can be used as a bioassay for olfaction (Murphy *et al.* 2001).

Study Objectives:

In this chapter, the ventilatory response behavioural bioassay is used to explore the relationship between reproductive behaviour and olfactory mediated intra-specific communication using putative pheromones and urogenital extracts. This was done by comparing results from animals that have undergone olfactory sensory deprivation with

sham-treated animals. A small set of studies have provided the basis of pheromonal communication in gobiids. These indicate that there is chemical communication between the males and females during reproductive activity (MacGinitie 1939; Tavolga 1956; Colombo *et al.* 1980; Murphy *et al.* 2001). In addition, Kulikova (1985) observed that the characteristic breeding colour and courtship behaviour observed in male round gobies was noticeable with abdominal enlargement in the females, suggesting that there may be chemical communication between males and females.

Putative pheromones estrone (E1) and ethiocholanolone (ETIO) were used to determine if previously reported increases in ventilation rates were due to olfaction (Murphy *et al.* 2001). The null hypothesis was that osmic and anosmic male and female round gobies would respond equally to putative pheromones. As shown in Murphy *et al.* 2001, it was expected that both osmic males and females would respond significantly to E1 and ETIO respectively. Dose response experiments ranging from 10^{-8} to 10^{-12} M were set up to examine behavioural responses to putative pheromones over a broad concentration range during different seasons. It was expected that males and females would show an increased ventilatory response or would respond to lower concentrations of putative pheromones during breeding season (summer) compared to non-breeding season (winter). In amphibians, gonadotropin releasing hormone is increased during natural breeding season and increases the excitability of OSNs (Eisthen *et al.* 2000) .

Responses to putative pheromones in males creates a standard by which tissue extracts harvested from HCG injected gravid and non-gravid females can be compared. The null hypothesis was that osmic and anosmic male round gobies would respond to

tissue from gravid and non-gravid females equally. It is expected that males would show significant increases in ventilation to gonadal extracts taken from HCG injected females during the summer (May- June, 2002) months but not in the winter months (October to December, 2001) because field observations indicate that spawning by round gobies in the Detroit River occurs from spring until autumn (Corkum *et al.* 1998). This is also expected because during the summer months, the cells of the follicular epithelium change from squamous to cuboidal and cytomorphological features indicate that these cells have enhanced functional activity in gravid females, thus producing more sex hormones (Kulikova 1985). It is expected that round goby males will respond to the increase in sex pheromones produced by the female and not the HCG. This was tested using muscle tissue of fish injected with HCG and pure HCG compound introduced to the study tank.

Gonadosomatic index (GSI) is typically used as a measure of maturity (de Vlaming *et al.* 1982) and hepatosomatic index (HSI) is used as a measure of condition (Barton *et al.* 1988; Goede and Barton 1990). The null hypothesis was that there would be no difference in GSI and HSI values in male and female round gobies in the winter and summer months. It was expected that during the round goby's natural reproductive season, May to August, that GSI values will be higher in comparison to those fish surveyed in the winter months. Miller (1984) reported that male gobiids have a GSI value that ranges from 0.3 to 3.6% and females range from 12 to 25%. GSI values for gravid female round gobies in the lab were 18 to 21% (Kulikova 1985) and 8 to 9% in the Detroit River (MacInnis 1997). With increases in gonadal weight, it is expected that the HSI value may decrease as most of the fishes energy is being used for reproduction.

I examined two methods of olfactory sensory deprivation. The first, copper sulfate ($CuSO_4$) is an agent that renders fish anosmic or removes the fish's ability to perceive odours. This is a technique has been used in behavioural trials for olfactory sensory deprivation (e.g., Sorensen 2001), but may cause other adverse side effects to fish, thus impairing their ability to behave normally. The null hypothesis is that there will be no difference in the response to putative pheromones by round gobies treated with $CuSO_4$ and controls. However, fish treated with $CuSO_4$ may still respond to odourants because copper exposure does not entirely block responses to L-alanine in salmon (Winberg *et al.* 1992). Moreover, $CuSO_4$ treatments do not destroy all of the OSNs within a fish's olfactory organ (Saucier and Astic 1995).

The second sensory deprivation technique, nasal occlusion with Reprosil®, a dental impression material, was used to addresses the inefficiency and potential toxicity of $CuSO_4$. Experimental controls for the Reprosil® technique including plugging one nostril and examining round gobies responses to odours before and after Reprosil® application. Because fish use both nostrils to sample odours, it is expected that there should be no difference in the fishes ability to perceive odours with either one or no plugged nares as there should be no differences in responses to odours or basal ventilation from one trial to the next in round gobies which had sham deprivation treatments.

Sex pheromones may have important roles in the mating strategies of round gobies because fish are constantly immersed in their physical and chemical environment, and their sensory systems are in continuous interactions with environmental changes (Hara 1993). Knowledge of the ability of the peripheral olfactory organ to detect putative

and natural pheromones is key in understanding the unspoken intra-specific communication that occurs during the mating season of the round goby. Understanding the chemical language used by the round goby may help fisheries biologists manage round goby numbers within the Great Lakes.

MATERIALS AND METHODS

Round goby collections and housing:

Neogobius melanostomus (Pallas 1811) were collected from the Detroit River during the spring, summer, and fall of 2000, 2001, and 2002 by angling in the Detroit River (Windsor, Ontario, Canada) and by trawling in the Canadian waters of the western basin of Lake Erie. Round gobies were held in aerated coolers with a handful of aquarium salt for transport back to the lab and held overnight in river or lake water mixed with dechlorinated tap water. The following day, round gobies were sexed by examination of the genital papilla (Miller 1984). Male gobies have a long pointed papillae with a broad base and a narrow tip while in females, the papillae is short and blunt being broad at the base and tip (Charlebois *et al.* 1997). Male and female round gobies were housed separately for maximum response during behaviour trials (Tavolga 1956). Adult sexually mature males can be distinguished by their typical grey/black colouration and their enlarged cheeks (Nikol'skii 1963; Miller 1984). Mature individuals of both sexes can be determined by the thickness and length of the papilla, with the papilla being larger in mature individuals.

All animals were maintained under constant photoperiod (16L:8D) in holding tanks (205 L) with a flow-through dechlorinated tap water system (10-20°C) at the University of Windsor holding facilities. Round goby diets consisted of Nutrafin® fish flakes and zebra mussels (*Dreissena polymorpha*) when available. Aquaria contained gravel, an air stone and PVC shelters and all experimental procedures reported in this

study were in compliance with guidelines established by the Canadian Council of Animal Care.

Measures of maturity and fitness in males and females:

All fish captured and used for behavioural bioassays were weighed, sexed, and measured before the treatment. Total length and weight for males and females used in the summer (reproductive stage) and winter (non reproductive stage) were analyzed to determine weight-length relationships expressed by the equation:

Equation 2.0 $W = a L^b$

where *W* is weight, *L* is length , *a* is the intercept, and *b* is the slope. The graph parameters of *a* and *b* were estimated by linear regression after $\log_{10}$ transformation of the data.

Following the winter and summer behavioural biossays, fish of both sexes were euthanized using an overdose of tricaine methane sulphonate (MS-222) and their liver and gonads were removed. The liver and gonads for both males and females were weighed immediately after removal from the body. The equation for linear regression is:

Equation 2.1 $y = a + bx$

where *y* is the variable being calculated, *a* is the y-intercept, *b* is the slope of the line, and x is some measured variable.

Quantitative examination can yield useful information on the health and condition

of fishes. Methods of obtaining standardized data from dissection have been developed to facilitate comparison of fishes of the same species from different localities (Strange 1996). This has been demonstrated thoroughly with hepatosomatic index (HSI) and is particularly true in the case of the gonadosomatic index (GSI) (Goede and Barton 1990). The GSI, a ratio of gonad weight to body weight, describes the relative size of the ovaries or testes and is useful in indicating time of spawning and to compare maturity of individuals (Nikol'skii 1963: Kulikova 1985). The HSI, a ratio of liver weight to body weight, is used as a condition factor because a reduction in the HSI has been demonstrated in fish populations stressed by acidity, other chemicals. handling, and altered water flows.).

GSI assumes that the relationship between gonad weight is linear with a zero Y-intercept and that the coefficient of variation is the same for all body fish weights (de Vlaming *et al.* 1982). Summer and winter GSI values were calculated using the following equation:

$$\text{Equation 2.2} \qquad GSI = \frac{\text{Gonad Weight (g)}}{\text{Body Weight (g)}} \times 100$$

Hepatosomatic (liver) index (HSI) for male and female round gobies was calculated using the equation 2.3.

$$\text{Equation 2.3} \qquad HSI = \frac{\text{Liver Weight (g)}}{\text{Body Weight (g)}} \times 100$$

The HSI and total length of the round goby was analyzed using linear regression. This variable is an indicator of condition; values may decrease in starved fish or increase in

fish subjected to toxins (Strange 1996).

Ovaries were removed from the female fish in the winter and summer months and preserved in modified Gilson's fixative (see Appendix A). Separated eggs from the ovarian tissue were counted with the aid of a dissecting microscope. The diameter, at the largest part of the spherical shaped eggs, was measured for thirty random eggs using an occular micrometer. Only ovaries with ripe, yolked, and unovuated eggs were used to avoid underestimation of fecundity as some eggs may have been spawned or lost during handling of the fish (Heins and Baker 1993). The number of eggs was compared to the body weight and length of the fish using linear regression. The relationship between number of ripe eggs and gonad weight and egg size was determined using linear regression.

Sensory deprivation techniques (copper sulfate and Reprosil®):

Copper sulfate ($CuSO_4$) is a known agent for impairing chemoreception in fishes and rendering them anosmic (Hara *et al.* 1976; Saucier *et al.* 1991; Winberg *et al.* 1992; Julliard *et al.* 1993; Hansen *et al.* 1999). Round goby males were initially immersed in 10 ppm (10 mg/L) $CuSO_4$ (ACS261, BDH, Dorset, England) for 10 minutes, twice, 12 hours apart (Winberg *et al.* 1992; Sorensen 2001). After these treatments with $CuSO_4$, the fish appeared lethargic and most died within the first 24 hours after exposure.

Nasal occlusion techniques using petroleum jelly and cotton have been used for olfactory sensory deprivation (Wisby and Hasler 1954; Jahn 1969), however we selected a dental impression material for nasal occlusion. It was important to assess the

effectiveness and to find an appropriate control for the nasal occlusion using hydrophilic vinyl polysiloxane impression material (Reprosil®), by modifying the $CuSO_4$ procedure of Winberg *et al.* 1992. I compared the two techniques to ensure that the nasal occlusion was an appropriate sensory deprivation technique.

Round goby males were treated with $CuSO_4$ and exposed to a putative pheromone to compare this technique to olfactory sensory deprivation by nasal occlusion Ten male round gobies were anesthetized with MS-222 for 10 minutes and then exposed to 10^{-8} M estrone (E1) (E1274, Sigma-Aldrich, Milwaukee, WI) treatment and their responses were recorded. Following this, the males were anesthetized and exposed to 30 mL of 10 ppm (mg/L) $CuSO_4$ for 10 minutes. The $CuSO_4$ solution was directed into the nares using an intravenous unit attached to a 60 cc syringe containing the $CuSO_4$. A 23-gauge needle was held in the posterior nares with Play-doh®. These fish were then exposed to the 10^{-8} M E1 treatment again and the differences between the percent basal ventilation rates before and after the $CuSO_4$ treatment were compared using a Mann-Whitney U test. Average basal ventilation was compared between control and $CuSO_4$ treatment using a paired t-test.

Copper sulfate treatments are known to cause specific degeneration of all mature sensory cells as well as numerous immature neurons (Cancalon 1982; Julliard *et al.* 1993). Histological comparisons were performed on the nares of five of the round gobies treated with $CuSO_4$ and two untreated fish using immunocytochemistry with antibodies against G_{o} and G_{olf}, G proteins found on microvillar and ciliated olfactory sensory neurons, respectfully (see Appendix A for procedure). This analysis was used to confirm

the destruction of these signal transduction proteins by $CuSO_4$.

One nostril was plugged rather than two, to ensure that the Reprosil® treatment was not affecting the fishes ability to ventilate normally. In addition, trials with the same osmic fish were performed on two consecutive days to show that there is no change in response from one day to another. Behavioural bioassays using 10^{-8}M E1 were conducted on ten male round gobies, both before and after a single nostril was plugged with Reprosil®. Percent basal ventilation was compared using a nonparametric Mann-Whitney U test after the odourant was added. Average basal ventilation was also compared between the first and second trial using a paired t-test.

Ventilation bioassays on male round gobies to the putative pheromone E1:

I examined ventilation rates of osmic and anosmic male round gobies in response to five different concentrations (10^{-8} to 10^{-12} M final concentration) of E1, in the winter (September, 2001 to January, 2002) and summer (May to June, 2002). Ventilation rates were used as a measure of odour detection because, in some benthic fishes, water flow through the naris changes in synchrony with ventilation (Nevitt 1991). Also, because Melinkat and Zeiske (1979) showed that the opening and closing of the accessory nasal sacs moves water through the peripheral olfactory organ and is directly related to opercular beats. Murphy *et al.* (2001) were the first to use the ventilation assay as a means to assess olfactory response. In all behavioural experiments with males anosmic individuals from the summer and winter were pooled after a t-test proved that they were not significantly different.

Round gobies were deprived of olfactory senses by plugging both nasal cavities with Reprosil® (626170, DENTSPLY International Inc., Milford, Delaware) a hydrophilic vinyl polysiloxane dental impression material. Before plugging the fishes nose with the impression material, the fish were lightly anesthetized with MS-222, the Reprosil® base was diluted in a large weigh boat with 100% acetone, and the excess acetone was poured off. The orange coloured base was then mixed with the solidifier, and drawn up into a 3cc syringe. A 23-gauge needle was attached to the needle and was used to inject the orange impression into the posterior nares until the nasal cavity was filled. A sham procedure, including all of the steps for plugging the nose, minus the actual injection of Reprosil®, was performed on fish which were deemed osmic. All gobies were recovered from the anesthetic by injecting oxygenated dechlorinated water over the gills with a 3 cc syringe.

Prior to performing behavioural trials, osmic and anosmic round gobies were left to acclimate overnight in 5.5 L aquaria. Each aquarium contained an air stone and a ceramic nest (15 cm long x 11 cm wide x 5 cm tall) with a ceramic lid and a clear side to facilitate videotaping. Each behavioural trial lasted a total of 60 minutes with a 30 minutes pretest period and a 30 minute test period after the odourant was added. Ventilation (rate of operacle openings) was observed and counted by viewing the video tape for 15 seconds every 5 minutes for the 30 minute pretest period. Estrone (E1) was then added in 100 mL background dechlorinated water to obtain final concentrations ranging from 10^{-8} to 10^{-12} M (modified from Murphy 1998; Murphy *et al.* 2001). Following the application of putative pheromone, ventilation was recorded for 15 seconds

at 4, 7, 10, 15, 20, 25, and 30 minutes during the 30 minute test period. Ventilation rates were first recorded at 4 minutes after the odourants were added because (1) that gave the odour enough time to thoroughly mix in the tank (Appendix B) and (2) allowed the fish to adjust to the presence of the researcher and just respond to the odour. This method deviated from that of Murphy *et al.* (2001) as their method of odour delivery was different. After the behavioural trial was completed, fish were euthanized with an overdose of MS-222 and their liver and gonads were removed as described earlier.

Each of the ten E1 treatments (10^{-8} to 10^{-12} M; osmic and anosmic) were replicated ten times with the total number of male fish used being 100 individuals in the winter and 100 individuals in the summer. Ventilation rate was transformed into a percentage (percent basal ventilation) by expressing the response to the odours as a percentage of the mean basal ventilation rate (pretest value) which was measured for 15 minutes before the odourant was added. This is necessary as the ventilation rates for each fish varies. Percent basal ventilation was analyzed using a non-parametric Mann-Whitney U test due the low number of replicates (Dytham 1999). The basal ventilation, averaged over 15 minutes, of anosmic and osmic fish was compared using a t-test. A dose response curve was created using the mean percent basal ventilation for the 10 osmic fish for each concentration tested at 4 minutes after the putative pheromone was added. Linear regression was used to determine if there was a relationship between percent basal ventilation and concentration of the putative pheromone.

Behavioural responses of males to female conspecific extracts:

Round goby males collected and housed between September and December, 2001 were exposed to urogenital tissue extracts from female round gobies in the winter 2001 and males collected from May to June, 2002 were exposed to female extracts from summer 2002 to observe changes in ventilation. Tavolga (1956) showed that the frillfin goby (*Bathygobius soporator*) males display courtship behaviour when exposed to swabs of the urogenital area of gravid females. Gravid females captured in May and June of 2002 were considered reproductive when they had an extended abdomen after at least one day of holding.

For conspecific extract trials, female round gobies were injected in the peritoneum with human chorionic gonadotropin (HCG) (5 units/g fish weight) (C1063, Sigma-Aldrich, Milwaukee, WI). The entire urogenital area (including ovaries, eggs, and egg sacs) of females injected was removed 12-18 hours after injection. The tissue was ground slowly using an ice cold glass homogenizer (04368-27, Cole-Parmer, Anjou, Quebec) in 10 mL 95% ethanol stored in an ice bucket filled with ice. Ice is used for minimizing chemical breakdown. For the behavioural trials, osmic and anosmic males (plugged with Reprosil®) were exposed to 1000 µL (in 5.5L tank) of the urogenital extract of gravid (May-June 2002) and non-gravid females (September 2001 to January 2002) with methods and materials similar to that described for putative pheromones tests. Osmic males percent basal ventilation was compared to anosmic males using nonparametric Mann-Whitney U test.

Since females were injected with HCG, it was necessary to test an equal amount

of homogenized female HCG injected muscle tissue for ventilatory responses in males (N=10), to rule out the possibility that HCG was eliciting a response. The processing of HCG muscle tissue was similar to the genitalia described earlier, as muscle tissue was ground in a glass homogenizer in 10 mL 95% ethanol, on ice. The muscle homogenate was exposed to male round gobies (N=10) to test for ventilatory increases. To test for HCG directly eliciting ventilation responses, 100 μL of HCG was used as an odourant and behavioural bioassays (N=10) were performed as those trials used for putative pheromones, but using HCG as the odour.

Ventilation bioassays on female round gobies to the putative pheromone ETIO:

I examined ventilation rates of osmic and anosmic female round gobies in response to five different concentrations (10^{-8} to 10^{-12} M final concentration) of etiocholanolone (ETIO) (E5126, Sigma-Aldrich, Milwaukee, WI), in the winter (September, 2001 to January, 2002) and three different concentrations (10^{-8} to 10^{-10} M final concentration) in the summer (May to June, 2002). Etiocholanolone was used in this study because it had previously been shown by Murphy *et al.* (2001) that female round gobies showed significant ventilation rate increases and EOG responses to this steroid. Behavioural bioassays were performed similarly to those administered to males (adapted from Murphy 1998; Murphy *et al.* 2001). Seventy female round gobies, 7 replicates x 10 treatments, 5 osmic and 5 anosmic (10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M ETIO), were used for behavioural bioassays in winter months. Thirty females, 10 replicates x 3 treatments (10^{-8}, 10^{-9}, 10^{-10} M ETIO), were used in the summer months. As with males,

anosmic females were also produced by plugging their nostrils with Reprosil®.

ETIO was added to the tank 30 minutes after the trial began near the air stone to ensure circulation throughout the tanks. As with the males, ventilation rates were recorded at 5, 10, and 15 minutes before the odour was added and 4, 7, 10, 15, 20, 25, and 30 minutes after the odour was added. Four minutes was used as a starting point for ventilation measurement because this allowed the fish to calm down after human exposure and it allowed time for the odour to circulate to the fish (see Appendix B for dye test). Respiration values were calculated as a percent of the basal ventilation by dividing the ventilation rate for a given time by the average basal ventilation rate, (an average of the respiration rates at 5, 10, and 15 minutes before the odour was added). These data were analyzed using a Mann-Whitney U test. A dose response curve, analyzed using linear regression, was developed for gravid and non-gravid females and percent basal ventilation at 4 minutes after the odour was added was plotted against the concentration of the odourant.

RESULTS

Measures of maturity and fitness in male round gobies:

Male round gobies (N = 150) used for behavioural bioassays in the winter were mean (± S.E.) 99.8 ± 1.25 mm total length and 12.5 ± 0.5 g. In the summer round

gobies (N = 150) were 120.3 ± 1.82 mm and 25.1 ± 1.3 g. There was a significant linear length-weight relationship for round gobies in the winter ($r^2 = 0.545$, $p < 0.0001$) and summer ($r^2 = 0.972$, $p < 0.0001$) (Figure 11), so as the length increases, the fish become more robust. There was a significant difference ($F = 11.97$, $p = 0.0006$) between the slopes of these lines, indicating that fish may grow faster in the summer season.

The GSI describes the relative size of the testes and is useful for indicating the time of spawning (Strange 1996). There was no significant relationship between the GSI and the length of the round goby in winter but there was a significant relationship ($r^2 = 0.116$, $p < 0.0001$) between these variables in the summer (Figure 12 A). As expected, male round gobies used in behavioural bioassays in the summer had a statistically higher ($t_{(0.05, 268)} = 12.78$, $p < 0.0001$) GSI value compared with round gobies in the winter (Figure 12 B). GSI values were mean 0.23 ± 0.02 % (ranged from 0.007 to 1.86 %) in the winter and mean 1.38 ± 0.08 % (range from 0.02 to 3.4 %).

The liver is a vital organ and reflects the maturity and fitness of the fish. The relationship between total length and HSI showed a significant negative relationship in the winter ($r^2 = 0.072$, $p = 0.003$), indicating that as the HSI decreases as the total length increases for 150 male round gobies from September 2001 to January 2002 (Figure 13). There was no significant relationship between the variables in the summer ($r^2 = 0.023$, $p = 0.067$).

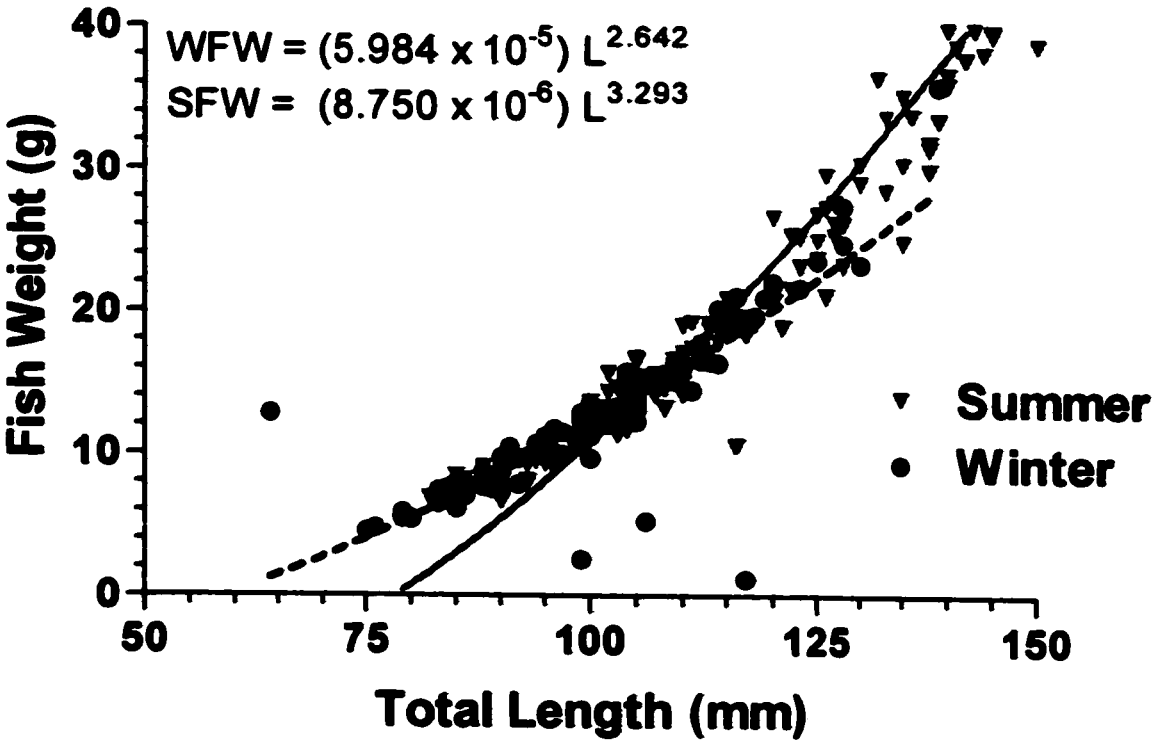

Figure 11: The relationship between fish weight and total length for 150 male round gobies collected in the winter (WFW) from September 2001 and November 2001 (r^2 = 0.546, $p < 0.0001$) and the summer (SFW) May and June 2002 ($r^2 = 0.972$, $p < 0.0001$). There was a significant difference ($F = 11.97$, $p = 0.0006$) between the slopes of these lines.

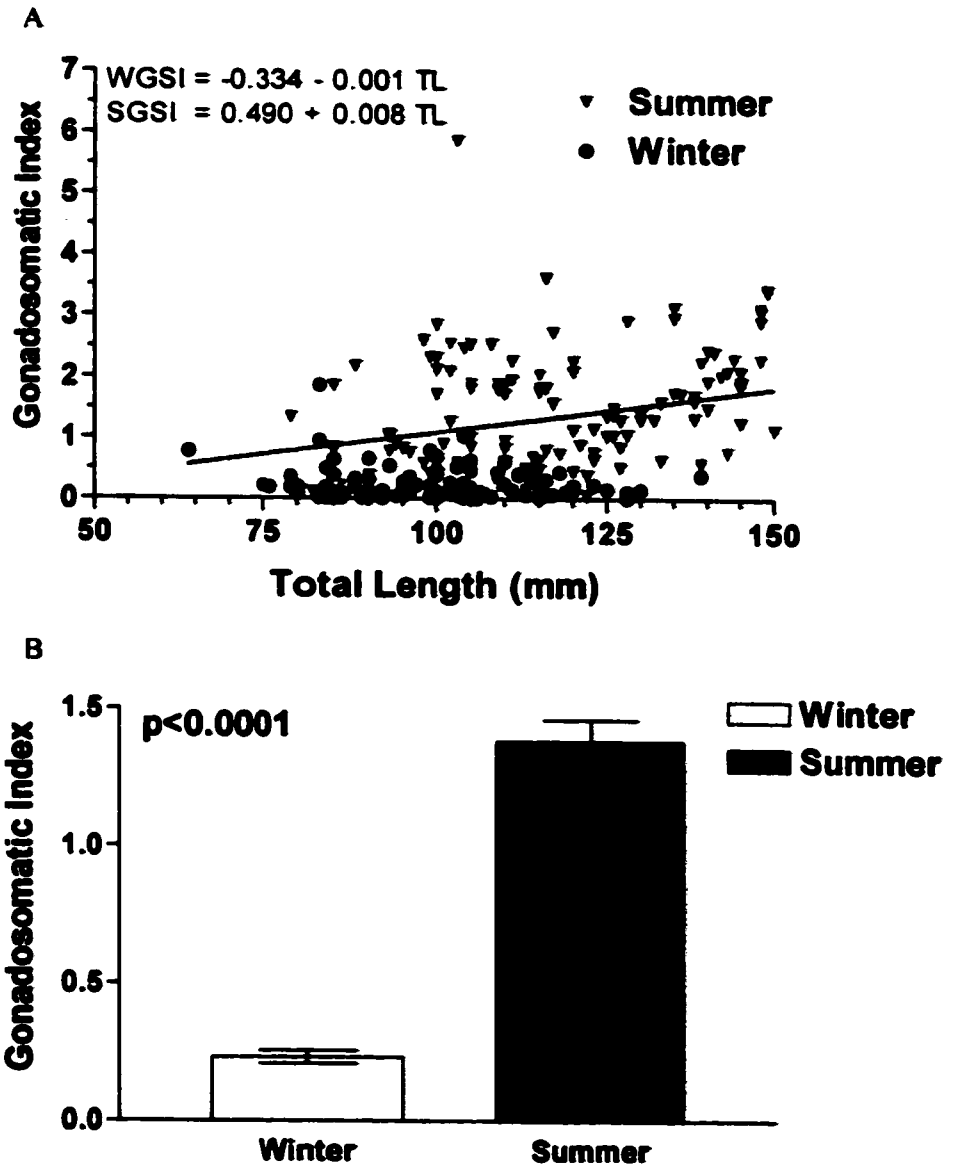

Figure 12: (A) Relationship between total length and gonadosomatic index in the winter (N=150) ($r^2 = 0.003$, $p = 0.573$) and the summer (N=150) ($r^2 = 0.116$, $p < 0.0001$) for male round gobies. (B) A comparison of GSI index mean (± S.E.) for male round gobies used for behavioural bioassays in the winter (N=150) and summer (N=150). The GSI was significantly higher in summer than in winter ($t_{(0.05, 268)} = 12.78$, $p < 0.0001$).

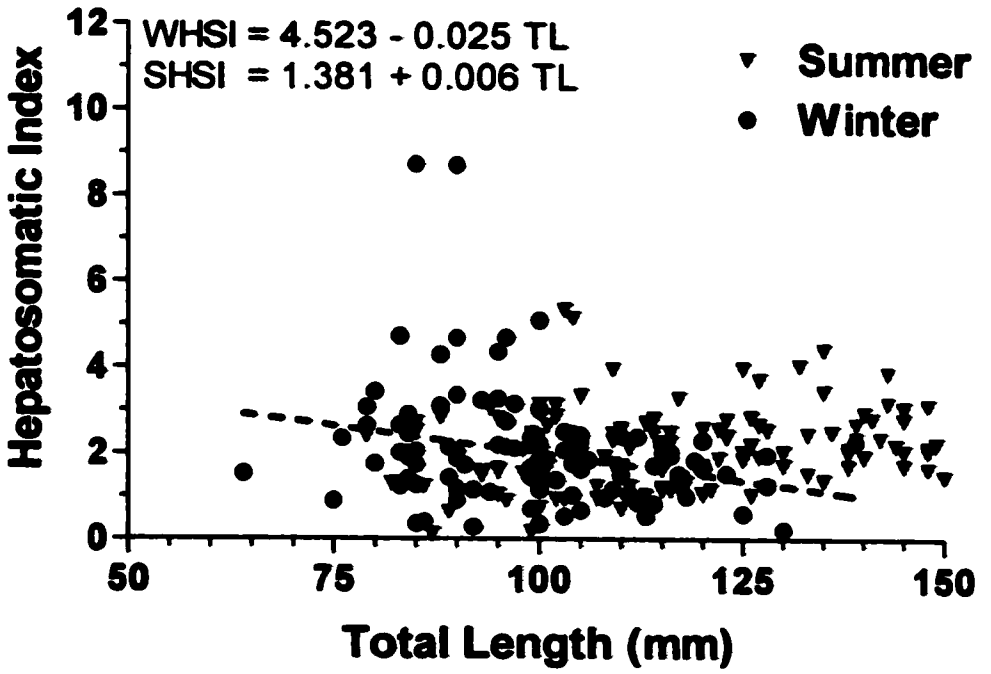

Figure 13: Relationship between total length and hepatosomatic index in the winter (WHSI) ($r^2 = 0.072$, $p = 0.003$) and summer (SHSI) ($r^2 = 0.023$, $p = 0.067$).

Measures of maturity and fitness in female round gobies:

Female round gobies (N = 70) used for behavioural bioassays in the winter had a mean (± S.E.) 84.6 ± 1.44 mm total length and 7.3 ± 0.40 g. In the summer round gobies (N = 30) were 94.0 ± 2.46 mm and 11.18 ± 1.07 g. in the summer (N = 30) (Figure 14). There was a significant weight-length relationship for female fish in the winter (r^2 = 0.835, $p < 0.0001$) and summer ($r^2 = 0.972$, $p < 0.0001$ summer) (Figure 14). The slopes of log converted regressions were statistically different ($F = 15.19$, $p = 0.0002$) when the regressions were compared with ANCOVA.

GSI in non-gravid female round gobies (Figure 15) was not significantly related to total length of the fish in the winter ($r^2 = 0.003$, $p = 0.703$) or in the summer ($r^2 = 0.002$, $p = 0.800$) (Figure 15 A). GSI values for female round gobies were higher in summer months (9.10 ± 1.21, ranged from 1.67 to 26.37) than the winter months (1.34 ± 1.57, ranged from 0.03 to 3.78) As expected, GSI values were significantly different in the winter compared to the summer ($t_{(0.05, 81)} = 8.354$, $p < 0.0001$) (Figure 15 B). Individual fecundity ranged from 2 to 766 with mean (± S.E.) 243.2 ± 20.80 eggs and was significantly correlated to total length in the winter ($r^2 = 0.345$, $p<0.0001$, $n = 54$) but not in the summer ($r^2 = 0.123$, $p = 0.443$, $n = 7$) (Figure 16 A). There was also a significant correlation between number of ripe eggs and weight in the winter ($r^2 = 0.303$, $p<0.0001$, $n = 54$) but not in the summer ($r^2 = 0.036$, $p = 0.685$. $n=7$) (Figure 16 B).

Though non-gravid female fish were used for behavioural bioassays during the winter, they did contain some ripe eggs. The number of ripe eggs was significantly correlated to gonad weight in the winter ($r^2 = 0.093$, $p = 0.033$, $n=54$) and in the summer

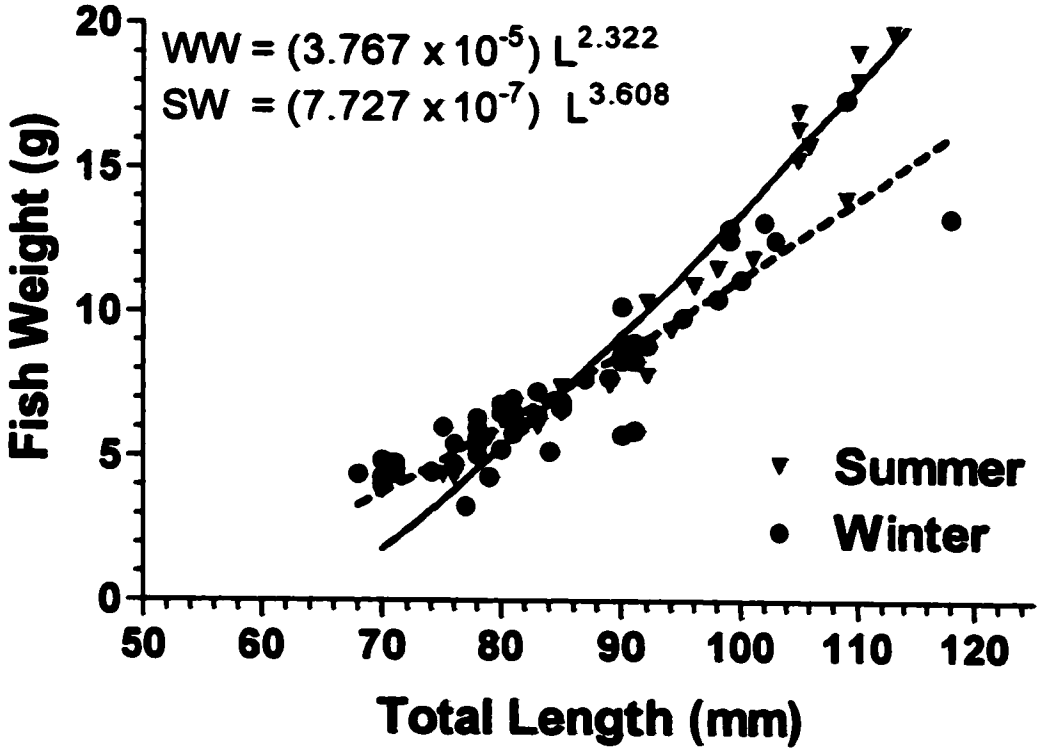

Figure 14: The relationship between fish weight and length for 70 female round gobies collected in the winter (WFW) from September 2001 and November 2001 ($r^2 = 0.835$, $p < 0.0001$) and the summer (SFW) May and June 2002 ($r^2 = 0.972$, $p < 0.0001$). There was a significant difference ($F = 15.19$, $p = 0.0002$) between the slopes of these lines.

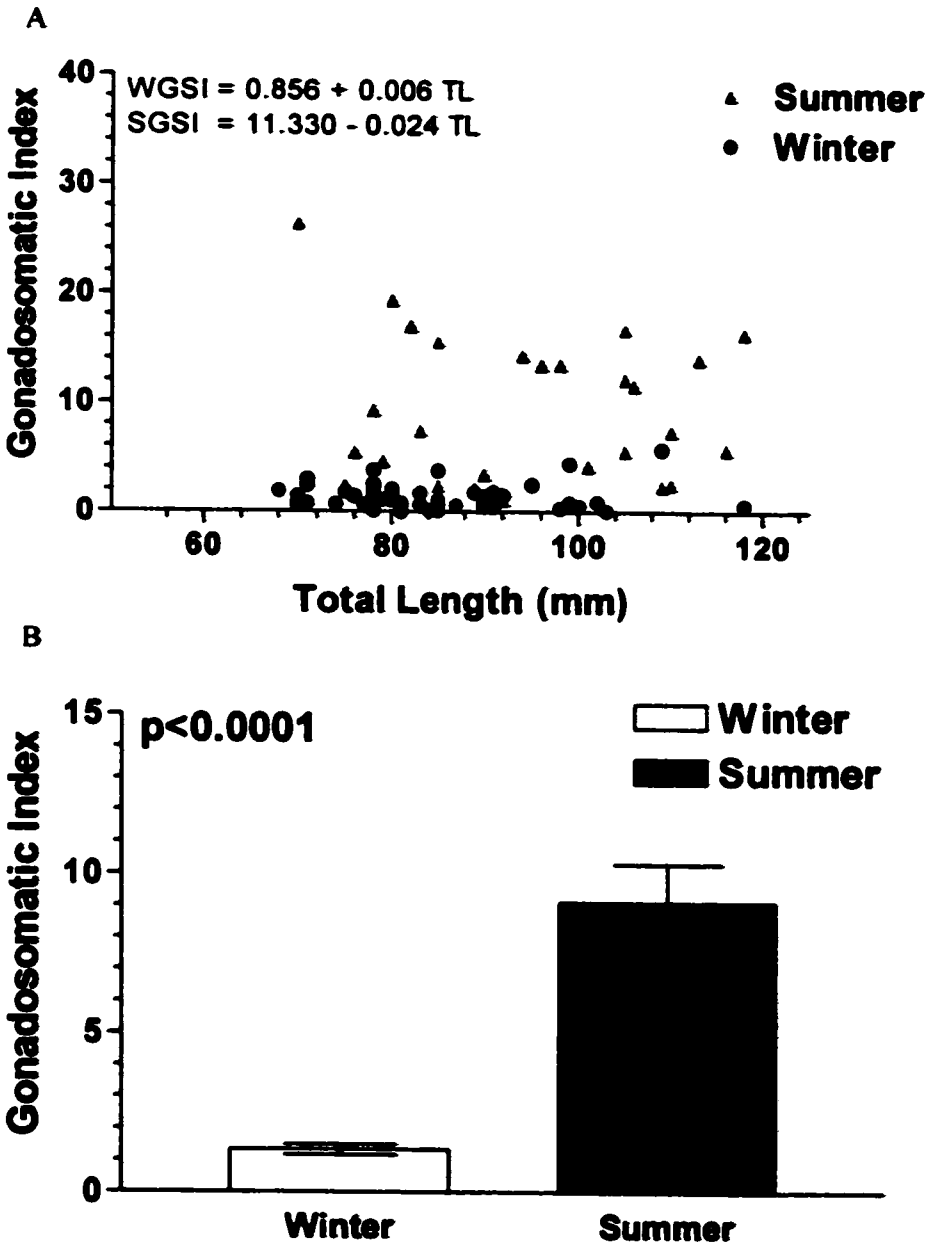

Figure 15: (A) The relationship between total length and gonadosomatic index for female round gobies in the winter (WGSI) ($r^2 = 0.003$, $p = 0.703$, $n=70$) and summer (SGSI) ($r^2 = 0.002$, $p = 0.800$, $n=30$). (B) GSI values in the winter months were significantly lower than in the summer months ($t_{(0.05,81)} = 8.354$, $p < 0.0001$).

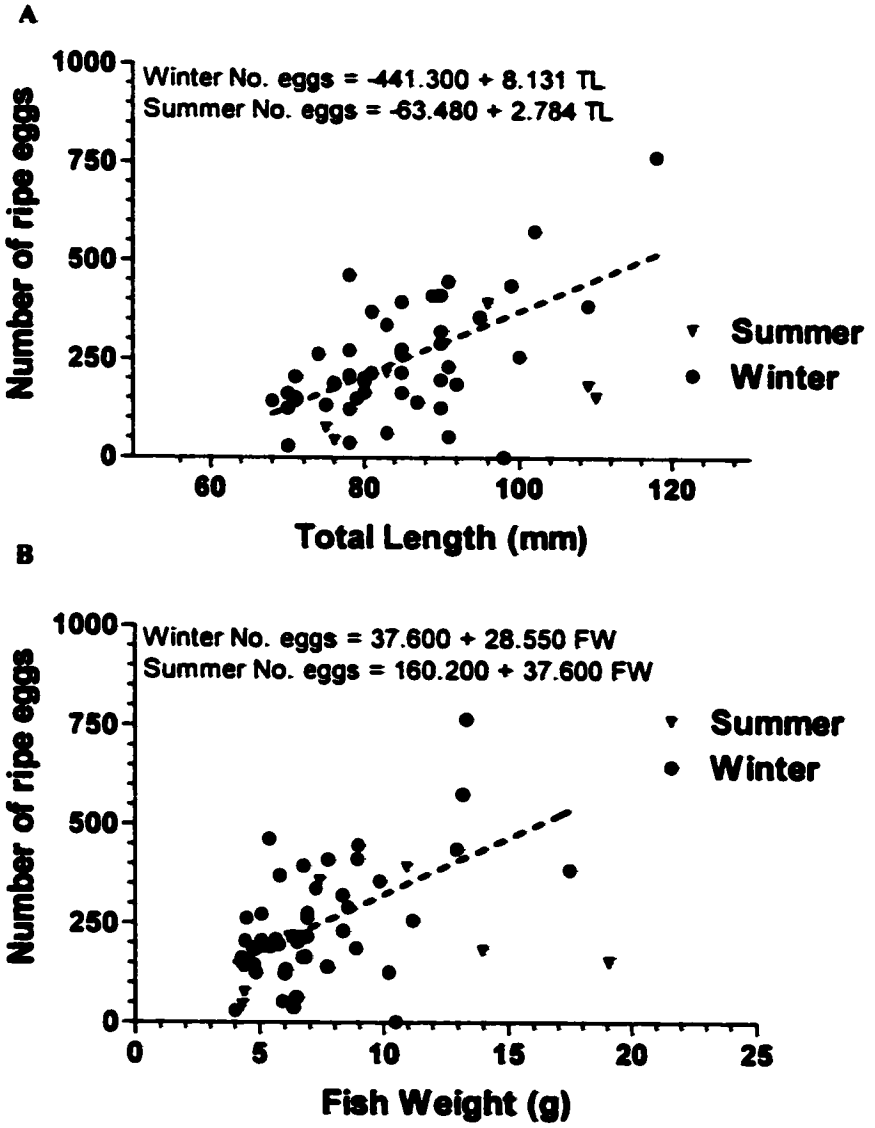

Figure 16: Relationship between: (A) total length and number of ripe eggs in the winter ($r^2 = 0.345$, $p<0.0001$, n=54) and the summer ($r^2 = 0.122$, $p = 0.443$, n=7) and (B) fish weight and number of eggs in the winter ($r^2 = 0.303$, $p<0.0001$, n=54) and summer ($r^2 = 0.036$, $p = 0.685$, n=7).

($r^2 = 0.839$, $p = 0.004$, n=7) (Figure 17 A), indicating that as the gonad weight increases, the number of ripe eggs increases. There was a significant linear relationship between the number of ripe eggs and egg diameter in the summer ($r^2 = 0.627$, $p = 0.033$), but not in the winter ($r^2 = 0.004$, $p = 0.673$) (Figure 17 B). There was a negative relationship between HSI and total length in the winter ($r^2 = 0.133$, $p = 0.007$) and the summer ($r^2 = 0.130$, $p = 0.050$) (Figure 18) meaning that as the fish gets longer, the percentage of the body that is liver decreases.

Sensory deprivation techniques (copper sulfate and Reprosil®):

The copper olfactory deprivation procedure of Winberg *et al.* 1992 was modified. Instead of immersing the fish in the $CuSO_4$ solution, intravenous units were pumped directly into the nostrils of the fish for sensory deprivation. Thus, I could be sure that any behavioural effects demonstrated by the round gobies were due to $CuSO_4$ in the nasal cavity and not by the toxic effects of $CuSO_4$ on the fish. Results showed that male round gobies treated with $CuSO_4$ responded significantly less ($p < 0.05$) to 10^{-8}M E1 after the treatment of 30 mL of 10 ppm $CuSO_4$ (Figure 19 A). The average basal ventilation rates, measured for 15 seconds and averaged over 15 minutes, were compared for round gobies during osmic and anosmic trials. There was a significant difference ($t = 2.813$, $p = 0.009$) in the average basal ventilation from the osmic and anosmic ($CuSO_4$) trials (Figure 19 B). This result strengthens the argument that a new fish should be used for each treatment, as there were no differences in average basal ventilation in the bioassays where new fish were used for osmic and anosmic trials (Figure 22 F).

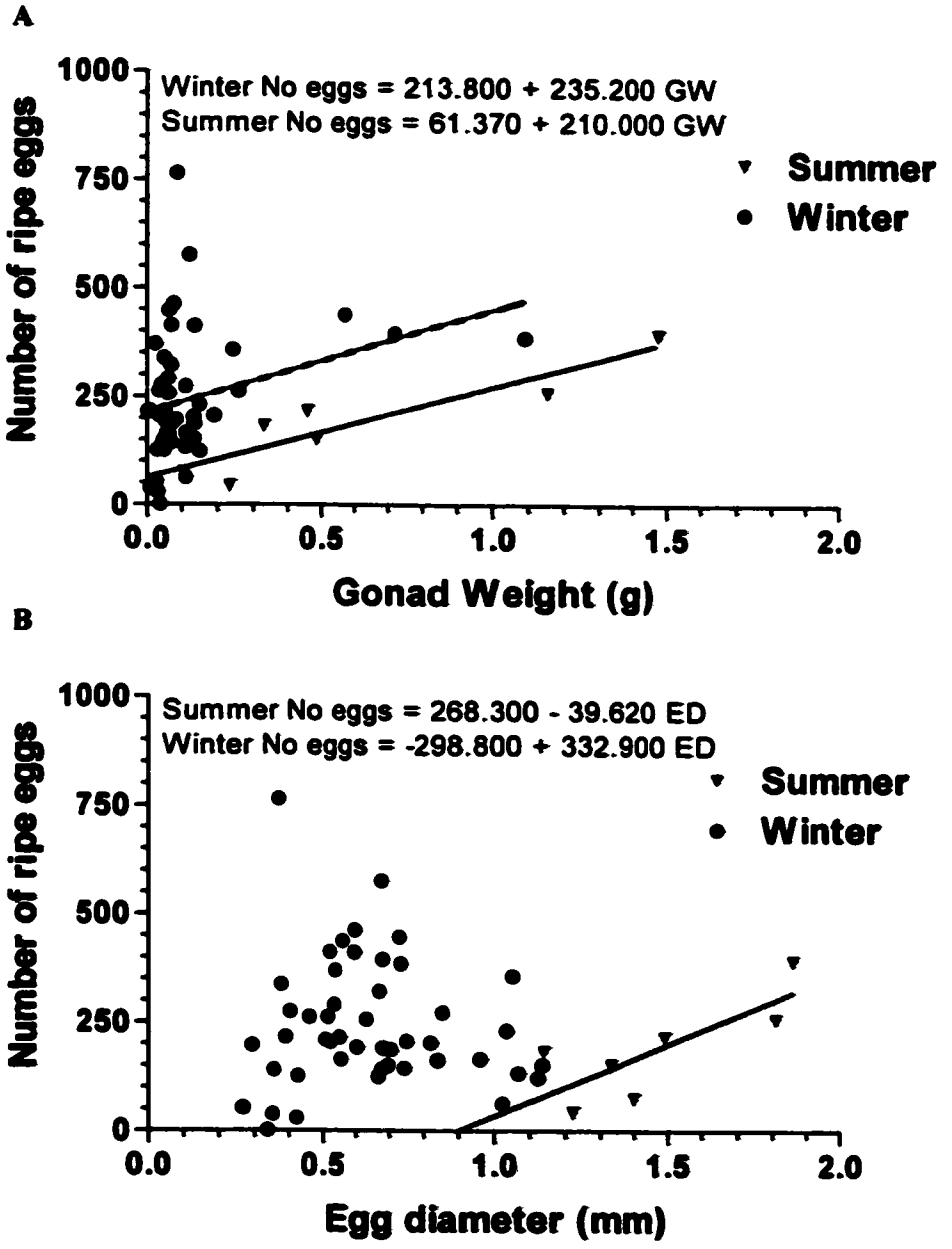

Figure 17: The relationship between: (A) gonad weight and number of ripe eggs in the winter ($r^2 = 0.093$, $p = 0.033$) and summer ($r^2 = 0.839$, $p = 0.004$) and (B) egg diameter and the number of ripe eggs in the winter ($r^2 = 0.004$, $p = 0.673$) and summer ($r^2 = 0.627$, $p = 0.034$).

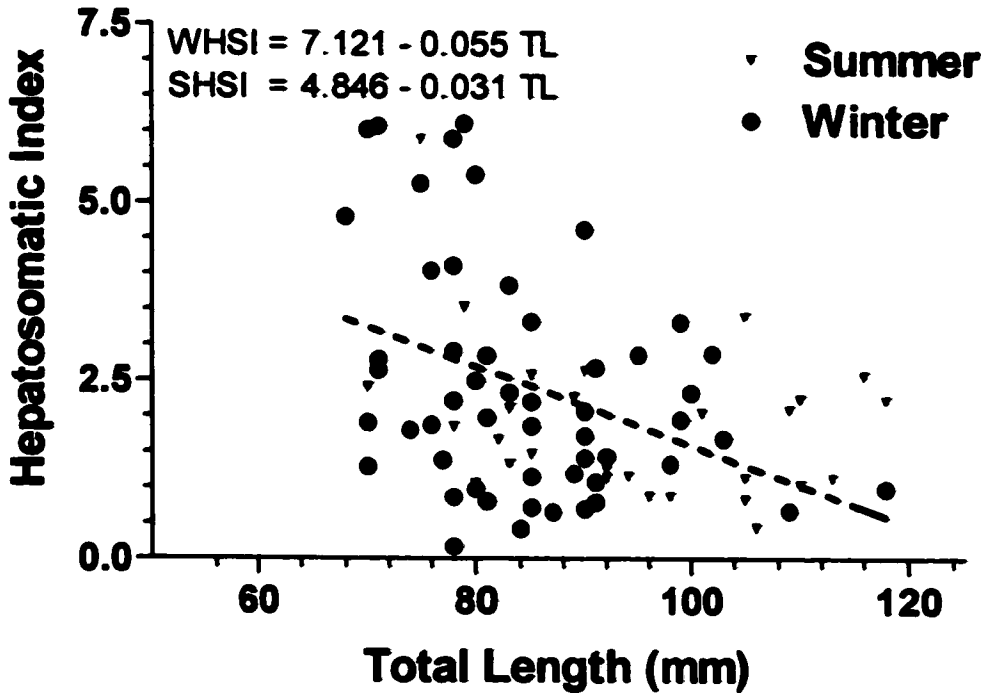

Figure 18: There is a negative relationship between total length and hepatosomatic index in the winter ($r^2 = 0.133$, $p = 0.007$) and summer ($r^2 = 0.130$, $p = 0.050$) for female round gobies.

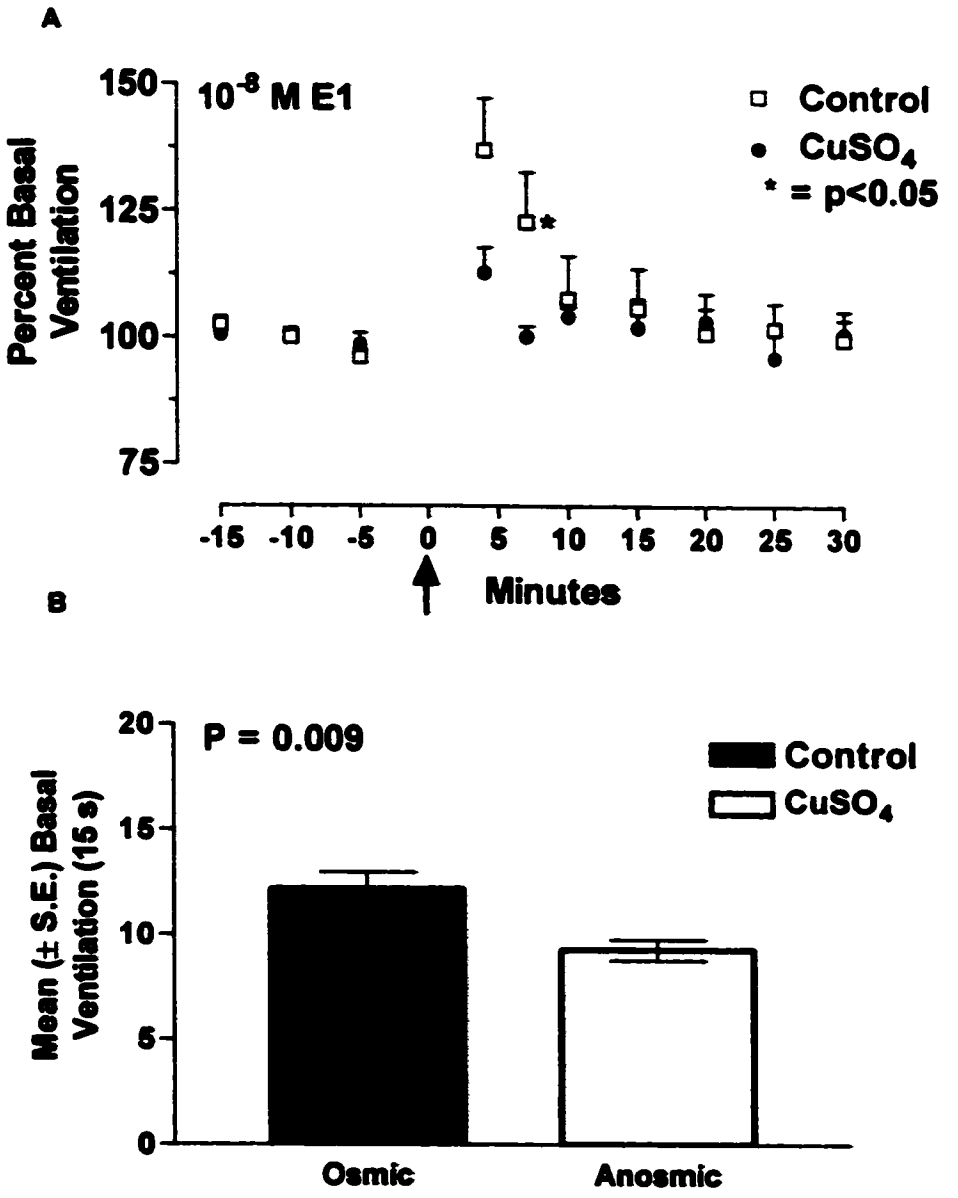

Figure 19: (A) Percent basal ventilation responses (mean ± S.E.) from to 10^{-8}M E1 (arrow) in males before and after treatment with 10 ppm copper sulfate (N = 10). (B) There was a significant difference ($t_{(0.05, 28)} = 2.813$, p =0.009) in the average basal ventilation in two consecutive osmic trial and anosmic trials.
Following nasal perfusion with 10 ppm $CuSO_4$ round goby olfactory epithelium

demonstrated a decrease in the staining intensity of G-proteins immunocytochemistry. Histological analysis using antibodies against $G_{\alpha olf}$ and $G_{\alpha o}$ showed that $CuSO_4$ is effective at destroying the G protein immunoreactivity, which likely impaired the fishes ability to perceive odours (Figure 20). The treated olfactory epithelium, when compared to an untreated fish of the similar size, contains less intact ciliated and microvillar sensory neurons. OSNs are sparsely distributed and appear to be degraded after copper exposure.

It was important to ensure that the Reprosil® treatment was not affecting normal ventilatory responses, so one naris was plugged as an appropriate control for this assay. For this, 10^{-8} M E1 was used, as it was the concentration that evoked the largest increase in basal ventilation. I found that even though the fish had one naris plugged, the round goby could still respond to odours (Figure 21 A). There was no statistical difference ($p>0.05$) between the male round gobies who had none or one of their nares plugged (Figure 21 A) and osmic fish from trial one to trial two (Figure 21 B). This means that when a fish was exposed to the odour on consecutive days, that there was still potential for a response to the putative pheromones had the fishes sensory system not been manipulated as in earlier control experiments.

There was no statistical difference ($t_{(0.05, 9)} = 1.546$, $p = 0.156$) between the average basal ventilation in the fish from one osmic trial to the trial with one naris blocked (Figure 21 C). However, there was a significant decrease ($p = 0.038$, $t_{(0.05, 9)} = 2.423$) in average basal ventilation from first osmic trial to the second osmic trial (Figure 21 D).

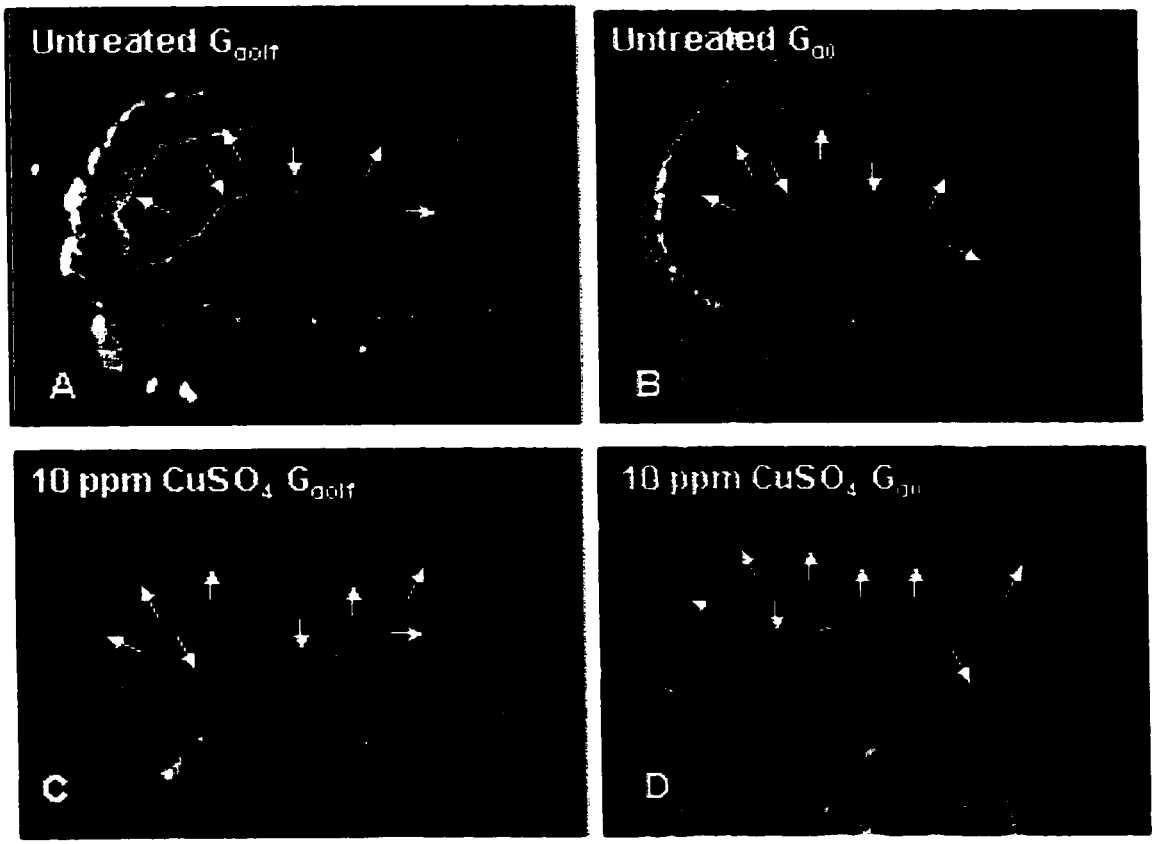

Figure 20: The effect of $CuSO_4$ on G protein immunoreactivity. A section of the peripheral olfactory organ shows intense $G_{\alpha olf}$ (A) and $G_{\alpha o}$ (B) immunoreactivity along the apical surface. Sections treated with 30 mL of 10 ppm $CuSO_4$ show little immunoreactivity after being treated with antibodies against $G_{\alpha olf}$ (C) and $G_{\alpha o}$ (D). Scale bar, shown in A, is 200 mm. The arrows point to the luminal surface of the olfactory epithelium.

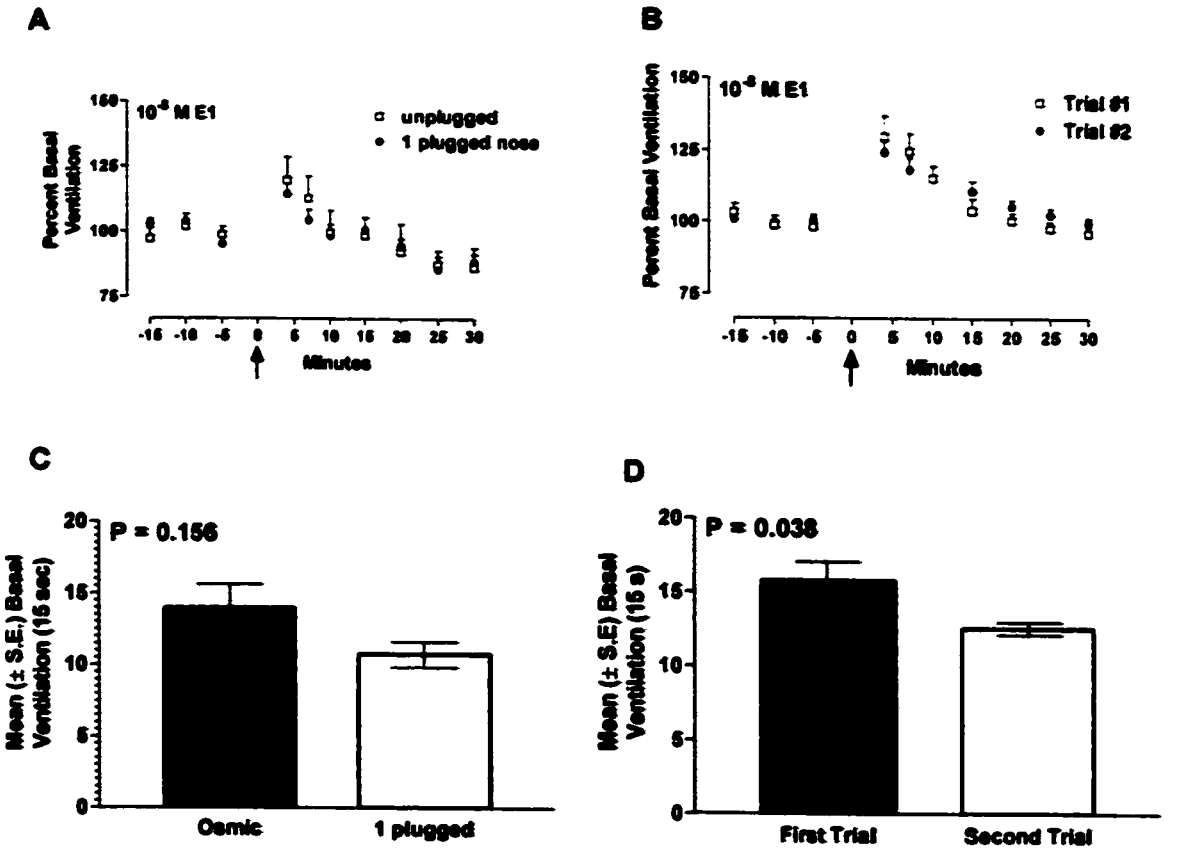

Figure 21: Percent basal ventilation responses (mean ± S.E.) in (A) osmic males and males with one nares plugged ($p>0.05$) and (B) osmic males with two consecutive treatments ($p>0.05$) (N = 10) before and after the odourant, 10^{-8} M E1 was added (arrow). Average percent basal ventilation was compared between (C) osmic males and males with one nares plugged ($t_{(0.05,9)} = 1.546$, $p = 0.156$) and (D) osmic males with two consecutive treatments ($t_{(0.05,9)} = 2.423$, $p = 0.038$).

Ventilation bioassays on male round gobies to the putative pheromone E1:

Behavioural responses to putative pheromones were measured using ventilation as in Murphy *et al.* (2001). Percent basal ventilation was compared between osmic and anosmic male round gobies (N = 10 per treatment) exposed to concentrations ranging from 10^{-8} to 10^{-12} M E1. Murphy *et al.* (2001) had previously demonstrated significant increases in ventilation and EOG responses (3 mV) in males round gobies to E1. Increased operacle beats, move water more quickly over the olfactory epithelium by the pumping action of the accessory nasal sacs (Melinkat and Zeiske 1979). Figure 22 (A. B) indicates that in the winter, male round gobies showed a significant increase ($p<0.05$) in percent basal ventilation to estrone at 10^{-8}M and 10^{-9}M, when compared to males following olfactory sensory deprivation. Response to the odour lasted between 7 and 10 minutes, after which ventilation returned to near basal levels. Mean (± S.E.) response at 4 minutes after the odour delivery was 146.0 (± 16.04) % basal ventilation for 10^{-8} M and 130.1 (± 4.80) % basal ventilation for 10^{-9} M. There was no significant response to concentrations of E1 at 10^{-10}M, 10^{-11}M, and 10^{-12}M (Figure 22 C, D, E) in the winter. In the summer, there was a significant increase ($p<0.05$) by males to E1 concentrations ranging from 10^{-8} to 10^{-11}M and responses to this odour lasted as long as 20 minutes at the higher concentrations. Mean (± S.E.) response at 4 minutes after the odour delivery was 133.7 (± 8.28) % basal ventilation for 10^{-8} M, 132.7 (± 9.10) % basal ventilation for 10^{-9} M, 112.4 (± 6.37) % basal ventilation for 10^{-10} M, and 116.8 (± 7.82) % basal ventilation for 10^{-11} M. In summary, males responded significantly to concentrations of E1 at 10^{-8} and 10^{-9} M in the winter and 10^{-8} to 10^{-11} M in the summer.

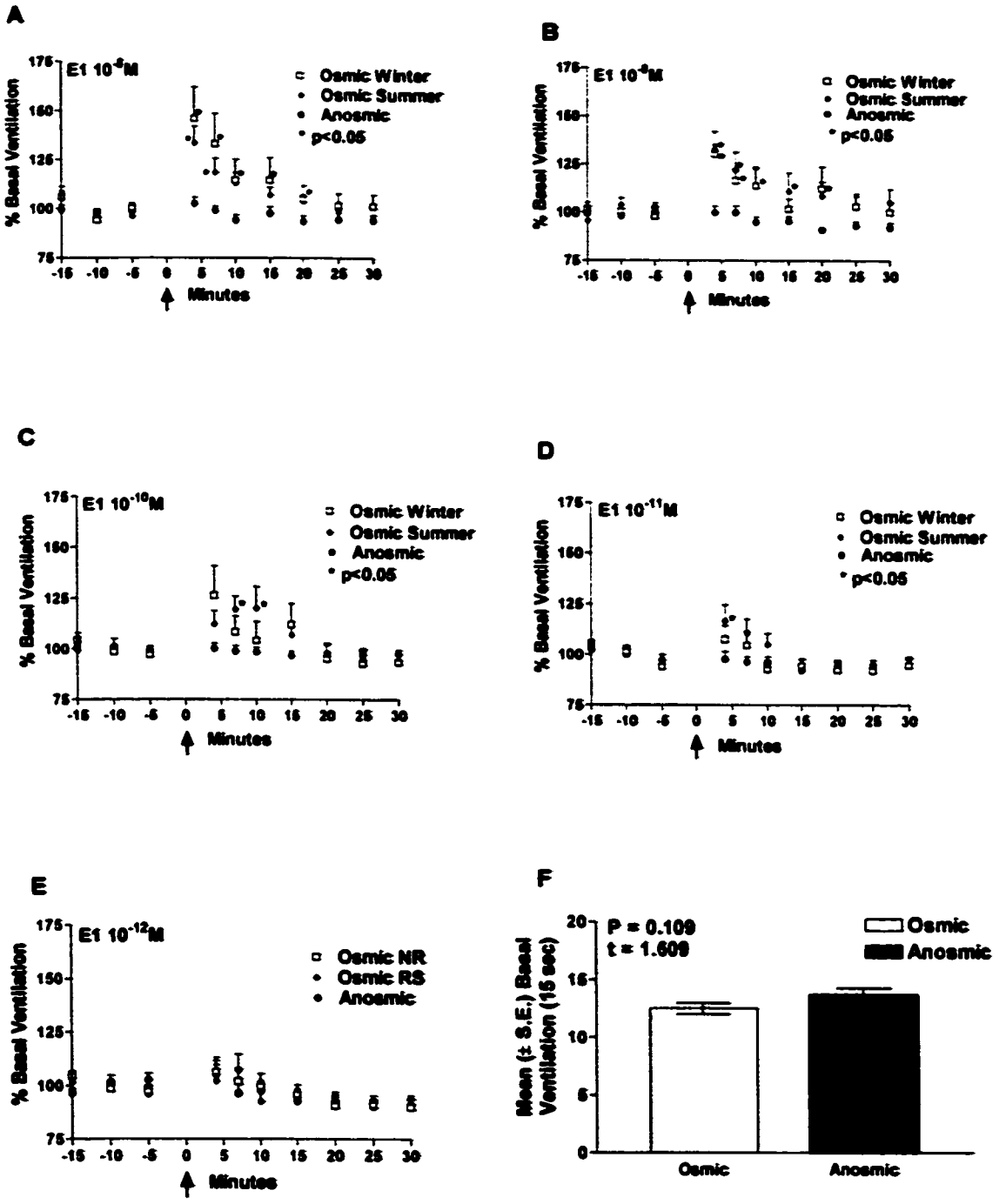

Figure 22: Percent basal ventilation (mean ± S.E.) responses in osmic (N=10) and pooled anosmic males (N = 20) before and after the odourant, E1 was added (arrow) at concentrations of (A) 10^{-8}, (B) 10^{-9}, (C) 10^{-10}, (D) 10^{-11}, and (E)10^{-12} M. * indicate a significance of $p<0.05$. Average percent basal ventilation was compared between osmic and anosmic fish (F) using a t-test ($p = 0.109$, $t_{(0.05,\ 198)} = 1.609$).

The average basal ventilation rate was calculated for each fish used in the behavioural trials, both osmic and anosmic. There was no significant difference ($p = 0.109$, $t_{(0.05,\ 198)} = 1.609$) between the average basal ventilation of the osmic and anosmic fish (Figure 22 F). These findings indicate that the Reprosil® plug had no effect on the normal ventilation of the fish and that normal ventilation was not affected by the plugs.

The relationship between concentrations of E1 and percent basal ventilation at 4 minutes after E1 was added was significant in the winter ($r^2 = 0.167$, $p = 0.005$, $n=100$) and summer ($r^2 = 0.177$, $p = 0.002$, $n=100$) meaning, as the concentration increases, so does the average percent basal ventilation (Figure 23). The graphs also show that after the concentration of the odourant decreases below 10^{-11} M, male round gobies can no longer significantly detect these odours.

Responses by males to female urogenital tissue extracts:

After establishing a baseline with putative pheromones known to induce ventilation rate increases (Murphy *et al.* 2001), it was important to assess the effects of natural pheromones on the ventilation responses in male round gobies. Osmic male round gobies did not demonstrate increases in percent basal ventilation to homogenized ovaries and egg sacs of females injected with HCG in the winter ($p > 0.05$), Mean (± S.E.) response at 4 minutes after the odour delivery was 125.5 (± 5.75) % basal ventilation. Males showed significant increases in the summer ($p < 0.05$) to the homogenate (Figure 24 A).

For control purposes, tissues of equal weight to gonadal tissue was used to test for

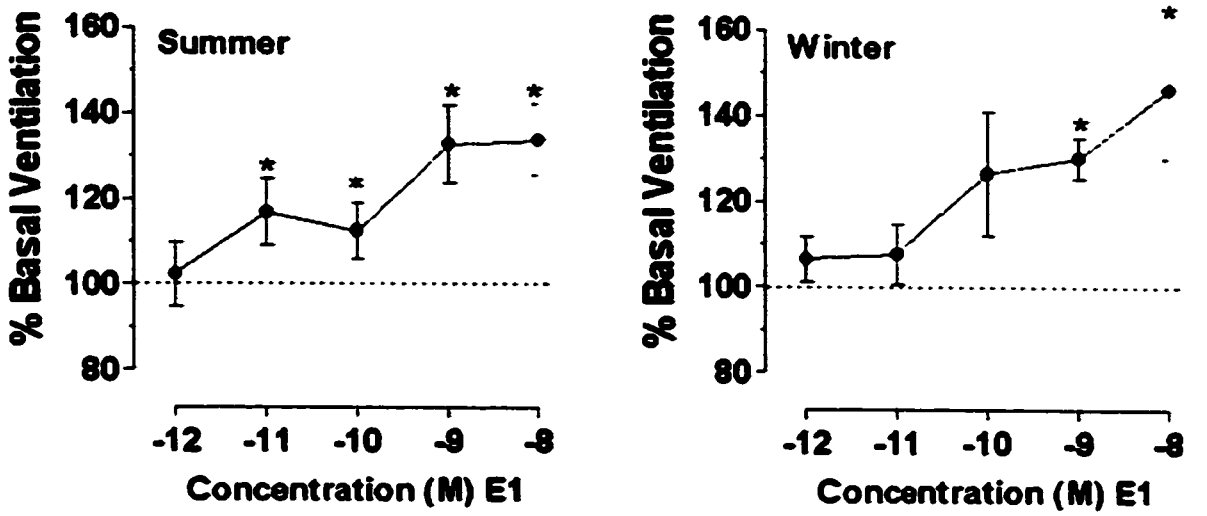

Figure 23: Dose response curve for percent basal ventilation (mean ± S.E.) for the winter ($r^2 = 0.167$, $p = 0.005$, n=100) and summer ($r^2 = 0.177$, $p = 0.002$, n=100) measured at 4 minutes after E1 was added to the tanks holding male round gobies. * represents $P<0.05$.

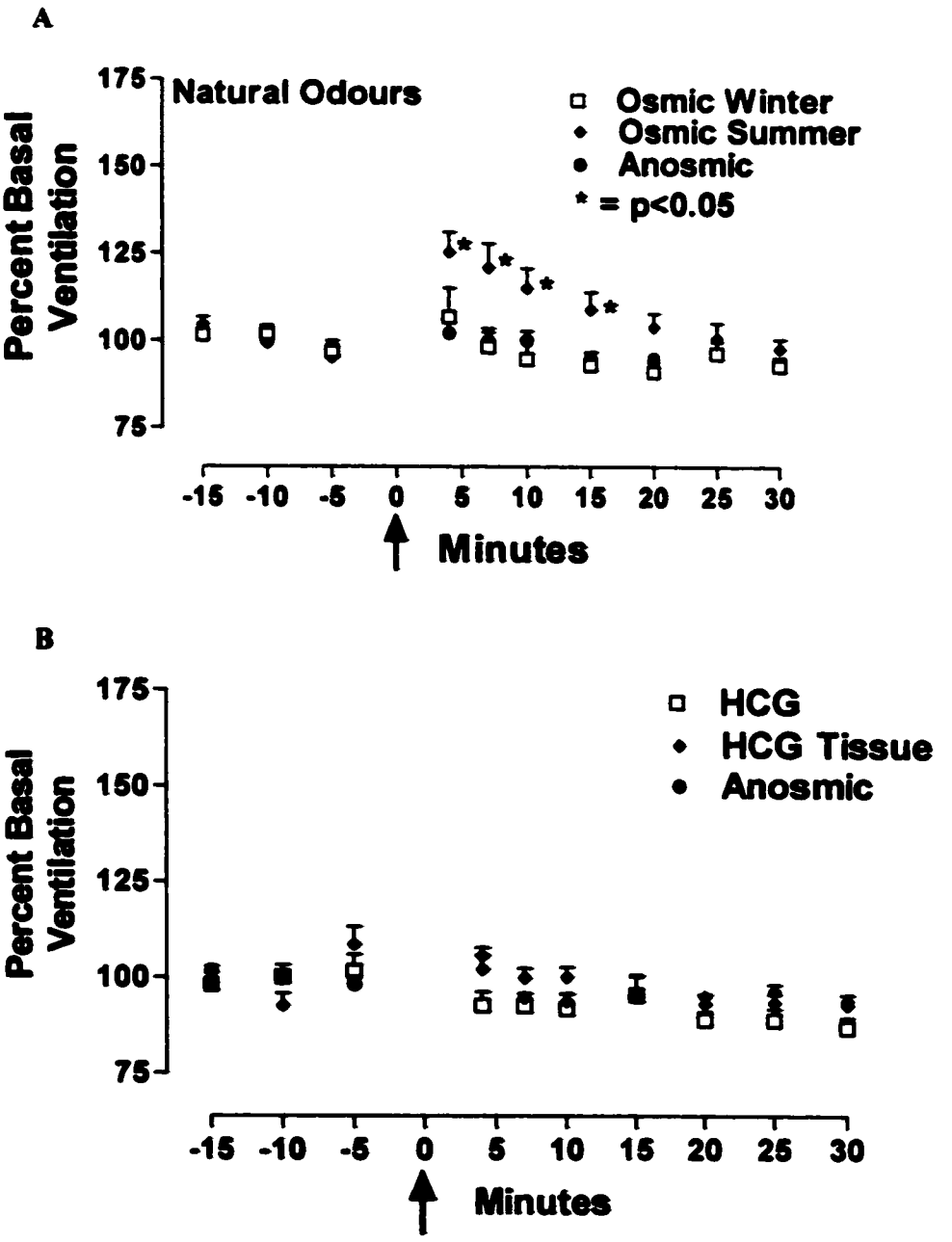

Figure 24: (A) Percent basal ventilation responses (mean ± S.E.) in osmic (N = 10) and anosmic males (N = 20) before and after the female odourant was added (arrow). * indicate significance ($p<0.05$). (B) Percent basal ventilation responses to HCG and muscle tissue from a female round goby injected with HCG.

responses to HCG as was 100 µL of pure HCG. Round goby males did not display basal ventilation increases to either of these treatments (Figure 24 B).

Ventilation bioassays on female round gobies to the putative pheromone ETIO:

Osmic female round gobies showed no significant responses during the winter to concentrations of ETIO (N = 7 per treatment) at 10^{-5} to 10^{-12} M (Figure 25) when compared to fish that underwent olfactory sensory deprivation. Osmic female round gobies showed a significant increase in percent basal ventilation to 10^{-5} M and 10^{-10} M (Figure 25 A & C) in the summer. Mean (± S.E.) response at 4 minutes after the odour delivery was 138.2 (± 7.30) % basal ventilation for 10^{-5} M and 128.8 (± 12.63) % basal ventilation for 10^{-10} M. A dose response curve (Figure 26) indicates that there is a positive slope, but no significant relationship in the winter ($r^2 = 0.025$, $p = 0.368$, $n=35$) or summer ($r^2 = 0.013$, $p = 0.558$, $n=30$) between percent basal ventilation at 4 minutes after ETIO was added. This shows that females are more responsive to odours in the summer however, there is no dose-response effect for females in response to ETIO.

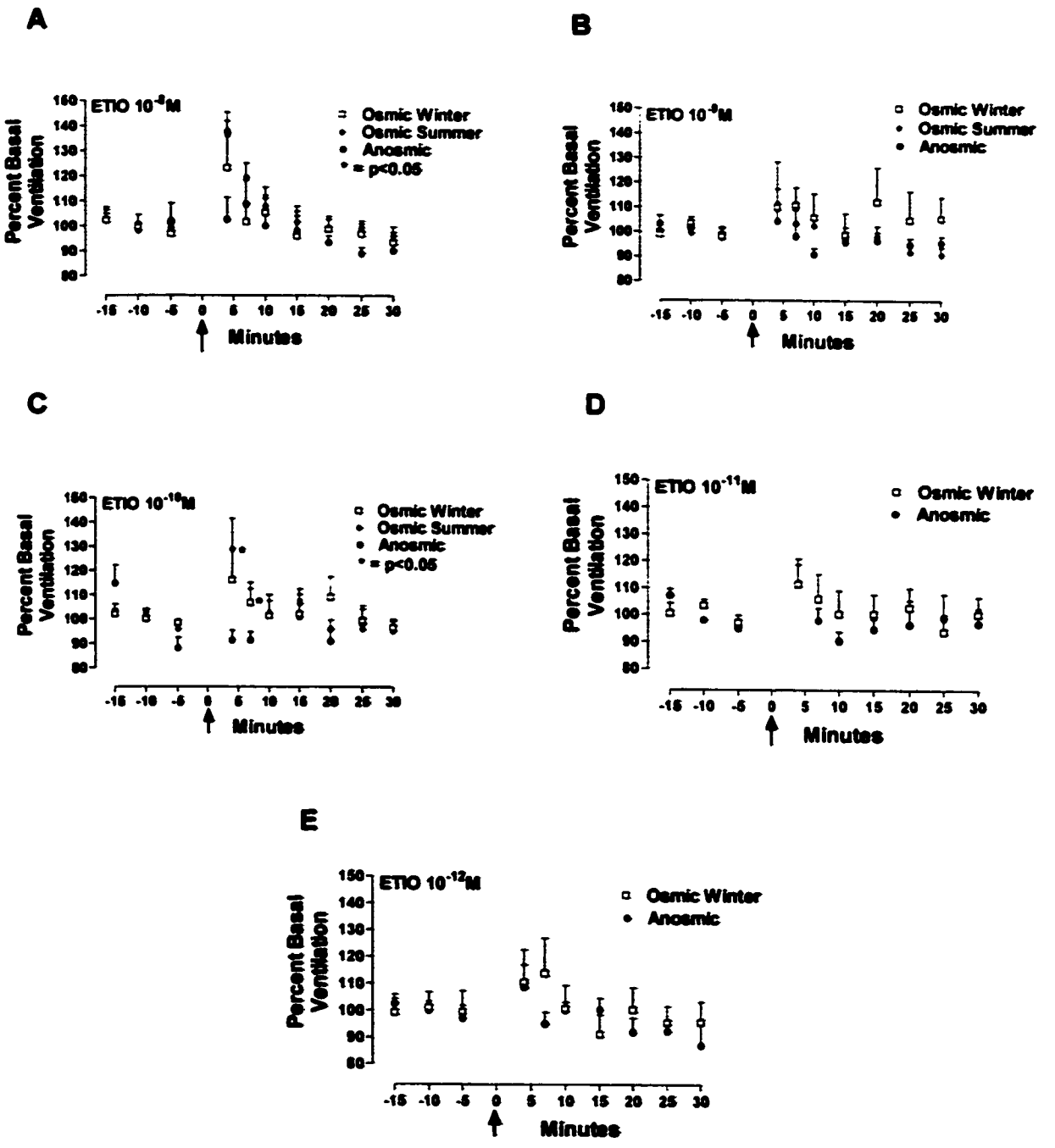

Figure 25: Percent basal ventilation (mean ± S.E.) responses in osmic and anosmic females before and after the odourant, ETIO was added (arrow) at concentrations of (A) 10^{-8}, (B) 10^{-9}, (C) 10^{-10}, (D) 10^{-11}, and (E)10^{-12} M. Female fish significantly ($p < 0.05$) responded to concentrations of 10^{-8} and 10^{-10} M in the summer only. * indicate $P<0.05$.

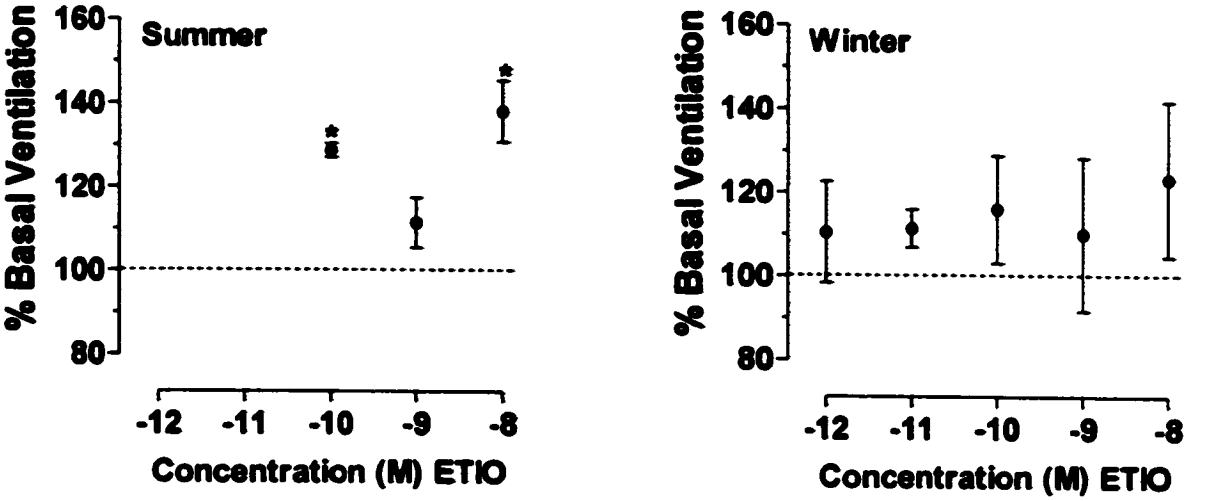

Figure 26: Dose response curve for percent basal ventilation (mean ± S.E.) in the winter ($r^2 = 0.025$, $p = 0.368$, $n=35$) and summer ($r^2 = 0.013$, $p = 0.558$, $n=30$) measured at 4 minutes after ETIO was added to the tanks holding female round gobies. * indicate $P<0.05$.

DISCUSSION

Measures of maturity and fitness in male and female round gobies:

Round goby capture results from the Detroit River and western basin of Lake Erie suggest that the population dynamics of the round goby in the Great Lakes are similar to round gobies found in its native region. In the Sea of Azov, the round goby's native territory, the landed commercial catch by trawling were average 130-160 mm TL for males and 110-150 mm TL for females (Berg 1949). The size of round gobies used in this study was similar with the largest male collected having a total length of 176 mm and a weight of 83.55 g. The largest female collected had a total length of 118 mm and a weight of 23.72 g. These numbers are higher than those reported by MacInnis (1997) where he states that the largest round goby male and female fish caught by trawl in the Detroit River were 124 mm TL and 112 mm TL, however these results were obtained only three years after the round goby had colonized the Detroit River.

Miller (1984) showed that male gobiids have a GSI range of 0.3 to 3.6%. I found that in round goby males that GSI values were mean (± S.E.) 0.23 ± 0.02 % (ranged from 0.007 to 1.86 %) in the winter and 1.38 ± 0.08 % (range from 0.02 to 3.4 %) in the summer. These results which are similar to Miller (1984), show that the GSI values vary seasonally with more reproductive effort being put forth in the spring/ summer months. Parental care in fishes is typically provided by males rather than females because energy saved in reproductive effort for females can be used for other types of offspring investment (i.e., larger clutch size) (Sargent and Gross 1993 cited in Corkum *et al.* 1998).

Therefore, female round gobies have higher GSI values in winter and summer compared to males.

In this study, the GSI values for female round gobies were higher in summer months (9.10 ± 1.21, ranged from 1.67 to 26.37) than the winter months (1.34 ± 1.57, ranged from 0.03 to 3.78) suggesting that female fish become sexually mature in the summer. The GSI value is a common tool used by fisheries biologists to estimate the maturity of fishes (Strange 1996; Goede and Barton 1990). In their native range, female round gobies have GSI values ranging from 0.79 to 2.15 % in September, 4 to 5 % in the winter and 18 to 21 % in the spring/ summer months (Kulikova 1985). These results suggest that female round gobies donate more of the energy toward reproduction in the spring and summer months. Thus, females will be more sexually active, producing and responding to hormonal pheromones in the summer.

Number of ripe eggs ranged from 0 to 766 in the winter with a diameter ranging from 330 μm to 1050 μm. In the summer, the number of ripe eggs was considerably smaller, 0-392, but the sizes were larger 1140 μm to 1860 μm. This is probably size related as the female round goby peritoneal cavity can physically only handle so many large eggs, so as the diameter of the eggs increases, the number of ripe eggs decreases. These finding are similar to those of Kulikova (1985) where range of female round goby oocytes was 100 to1200 μm in diameter with eggs in September being 572 ± 18 μm. Kulikova (1985) also showed that in female round gobies, as the GSI increases, mean diameter of eggs also increases (e.g. GSI 3.01% with egg diameter being 953 ± 7 μm). Largest egg diameters in Kulikova (1985) after HCG injections was 1000 to 2200 μm.

The average clutch size of their female round gobies after a dose of 25 and 50 IU HCG per gram of body weight was 300 to 450, but these numbers did not differ from the study control (Kulikova 1985).

In males and females there is a negative relationship, or slightly positive, between HSI and length. This relationship is only significant in the winter but the trend is typically negative. HSI may decrease with increasing fish length because, as the fish becomes larger, it presumably becomes more mature and therefore may be putting more of its energy into reproduction. Accordingly, the body, including the vital organs, may become smaller in proportion to the fishes body size. Thus, this condition factor is highly variable and less accurate than gonadosomatic indices (Strange 1996). Additionally, HSI may be significantly negatively correlated with TL in winter due to the higher occurrences of parasites (nematode cysts) found in the winter months (*pers. obs.*). Using liver as a representative of the maturity of a fish and condition is good if the fish liver is not damaged by environmental factors or reduced in size due to an overload of parasites (Barton *et al.* 1988; Goede and Barton 1990).

Sensory deprivation techniques:

Immunocytochemistry using antibodies against $G_{\alpha olf}$ and $G_{\alpha o}$ showed that $CuSO_4$ treatment invariably causes neuronal cell damage, prominently at the apical surface. Julliard *et al.* (1993; 1996) and Starcevic and Zielinski (1997) previously showed that compared to untreated cells in rainbow trout (*Oncorhynchus mykiss*), copper-treated cells show fewer cilia, damaged cell membranes, and are irregular in their general morphology.

This study was the first to show that there is a loss of G proteins, $G_{\alpha olf}$ and $G_{\alpha o}$, needed for olfactory signal transduction. Previous behavioural tests of copper sulfate treatment in Hawaiian gobies (Sorensen 2001) showed that when a similar copper sulfate treatment was applied, fish no longer responded to homing odours.

Copper exposure leads to behavioural changes, which are regulated by the olfactory system. Saucier *et al.* (1991) demonstrated significant decreases in attraction to rearing water in rainbow trout after chronic exposure to sublethal copper sulfate. The results of Sorensen (2001) were similar to those of Saucier *et al.* (1991) where he showed that Hawaiian gobies treated with $CuSO_4$ did not respond to migratory stimulants in a Y maze. Likewise, I observed that male round gobies that were treated with $CuSO_4$ showed a decreased response to E1 at 10^{-8} M, a known ventilatory stimulant (Murphy *et al.* 2001).

Although $CuSO_4$ was an effective agent for olfactory sensory deprivation, fish immersed in two, ten minute treatments of 10 ppm $CuSO_4$ became lethargic and often died (*pers. obs.*). This suggests that $CuSO_4$ may have toxic side effects in fish and the toxicity of copper has been shown in numerous fish studies (e.g. review by Atchison *et al.* 1987; Weis *et al.* 2001). The data in this study suggests that plugging both nares with dental impression material is a more effective and less detrimental treatment for olfactory sensory deprivation because round gobies treated with copper still show some increases in basal ventilation to E1 at 10^{-8} M. Copper sulfate is known to damage OSNs but it does not destroy all of the sensory function as fish still have slight EOG responses after $CuSO_4$ treatment (Winberg *et al.* 1992), suggesting that all of the OSNs were not damaged during treatment. There is still residual G protein staining in fish olfactory epithelium

treated with copper sulfate suggesting that olfactory signal transduction can still occur.

It may be suggested that an effective control for testing a fishes response to an odour would be to use the same animal for two trials, one where fish was osmic, and the second where they are sensory deprived, in order to compare the two treatments. This study shows that this is not an effective way to measure olfactory behaviour responses to odours as the fishes basal ventilation significantly decreases after the first trial. Perhaps the fish was more accustomed to the test tank. It has also been shown by Chivers *et al.* (2000) that if the reticulate sculpin (*Cottus perplexus*) is deprived of food for 2 days, they fail to respond to conspecific alarm cues. Food deprivation is necessary in these treatments as food odours may conflict with responses to putative pheromones and natural odours. Therefore, fish used in repeated trials would not respond to odours as effectively in the second treatment and I showed that basal ventilation was significantly less on the second trial day in three of the four control studies.

Responses to putative pheromones in male round gobies:

Murphy *et al.* (2001) was the first to show that a perciform, the male round goby, responded by EOG and with ventilation increases to estrogen and conjugated estrogens. In a review, Specker and Sullivan (1994) suggested that during ovarian development estrogenic compounds (e.g., E1) produced by the ovary may be released in free and conjugated forms, to function as pheromones inducing nest-building, courtship, and male gonadal development. Males should be tracking these chemical signals throughout all seasons since its seems the reproduction season is tracked and initiated by female round

gobies (Moiseyeva and Rudenko 1976; Kulikova 1985). I showed that males perceive estrogens at concentrations of 10^{-8} and 10^{-9} M in the winter and summer. Males may synchronize their territoriality and nest-building with female reproductive development by detecting estrogens produced and released during vitellogenesis (Specker and Sullivan 1994).

Apparently both the black goby (Colombo *et al.* 1982) and round goby males may initiate courtship by releasing an attractive chemical (e.g., ETIO) to females. Thus the female switches from a pheromone sender to pheromone receiver. Although male round gobies can sense odours at lower concentrations in the summer (10^{-10} and 10^{-11} M) compared to the winter (10^{-9} M), they must sample for mating cues throughout the year because they might not be as sensitive to changes in photoperiod or temperature as females. Females are suspected to chemically initiate reproduction. Increased response to a pheromone may reflect a general increase in olfactory responsiveness induced by physiological changes associated with reproduction (Partridge *et al.* 1976; Eisthen *et al.* 2000).

Evidence from male teleost reproductive chemical signaling systems, and this study suggest that the chemical compound(s) that may be released by male round gobies for the purpose of attracting females to nest sites, are likely a number of free and conjugated androgens (Murphy 1998; Murphy *et al.* 2001). Since ETIO-g has been identified in the black goby as a potential pheromone which attracts females and stimulates oviposition, ETIO-g (or ETIO) may also have pheromonal function in female round gobies. The pheromone may be released via the urine (Colombo *et al.* 1982), or by

secretions from the cement gland, a gland located in the genital area, onto substrates in preparation for female oviposition (Tavolga 1954; Ota *et al.* 1996). It is possible that females respond to this ETIO(-g) to find a suitable nest site and a spawning male.

As in other gobiids (Magnhagen and Kvarnemo 1989; Forsgren 1997), male round gobies may possess alternate mating strategies, with Type I males guarding nests and exhibiting parental care, and Type II males showing sneaker traits. This is based on field observations reported in MacInnis (1997) that two male morphs exist in the round goby. Type I males have a GSI of ~3%, are larger (> 80 mm) and possess male secondary traits such as enlarged cheeks and black colouration. Type II, or sneaker males, are similar in appearance to females, have larger testes (GSI ~ 4%), are typically smaller in length (< 70 mm), and have greatly extended urogenital papilla with reduced cement glands (Magnhagen and Kvarnemo 1989; Forsgren 1997). If two morphs exist, sneaker males may respond to ETIO to find a potential Type I male's nest site and wait nearby to fertilize the eggs of females. Murphy *et al.* (2001) showed that males respond to ETIO and ETIO-g, however not as much as females.

During courtship and spawning at the nest site, males may respond to E1, released continuously by ovulated females. Male round gobies are aggressive nest guarders and will quickly eliminate intruders from the nest site (*pers. obs.*; MacInnis and Corkum 2000). The E1 pheromone may be particularly important within a dark nest, where visual cues might be ineffective. This estrogen compound may also enable Type I males to discriminate between Type II males and females, blocking the Type II males from entering the nest and fertilizing or foraging on deposited eggs.

Approximately half of the fish species tested, using EOG and behaviour assays, have been found to detect one or more gonadal steroids at sub-nanomolar concentrations (review by Sorensen and Caprio 1998). The dose-response reaction typically never saturates because as the concentration of the odourant increases, the trigeminal innervation system is invoked, and the odourant becomes an irritant and will elicit other behaviours (Finger *et al.*1990 ; Silver 1990). If the stimulus is too intense, an animal may respond with other behaviours. Nonolfactory trigeminal (Gasserian) nerves are located in all epithelium including olfactory epithelium and have been shown on the apical surface of olfactory epithelium in rats, tiger salamanders (*Ambystoma tigrinum*), and in all anurans studied (e.g. bullfrogs, *Limnodynastes dumerili*, and grass frogs, *Limnodynastes tasmaniensis)* (Bouvet *et al.* 1987; Finger *et al.* 1990). Trigeminal nerves have also been observed in the sea lamprey (B. Zielinski *pers. comm.*) The peripheral olfactory organ has been described as similar in structural characteristics as another anuran, *Xenopus laevis* (Belanger *et al. in press*) and thus round gobies may possess trigeminal nerves. However this trigeminal system may not be invoked in the round goby due to nasal occlusion and the blocking of these receptors.

Responses by males to female conspecific tissue extracts:

Osmic male round gobies responded with increased ventilatory behaviour to extracts of homogenized gonadal tissue induced with HCG during the summer months when reproduction is known to occur in the Detroit River (MacInnis and Corkum 2000). As well, a change in basal ventilation of a single male from 48 bpm to 212 bpm was

witnessed when a known spermiated male was placed in a tank where a female had just oviposited in our wet lab facility (*pers. obs.*). Kulikova (1985) stated that during the spring and after HCG injections, female round gobies with a marked swelling of the abdomen induced characteristic breeding colour in the males and typical courtship behaviour was witnessed after that. It is believed that the stimulative effect of HCG undoubtedly occurs by increasing the synthesis of the steroid sex hormones, a phenomenon that occurs naturally in uninduced females (Kulikova 1985). Tavolga (1956) showed that a guarding male, in breeding season, responded to a gravid female gonadal swabs by a characteristic colour change and locomotor behaviour of fanning as well as gasping. Pheromones are known to also stimulate conspecific males as attractants in the sea lamprey (Teeter 1980), catfish (Timms and Kleerkoper 1972), and the guppy (Grandolfi 1969).

There have been numerous attempts to identify the source and chemical nature of sexual pheromones in fish. It has long been know that some substances originating from the gonad have pheromonal activities in eliciting courtship behaviour in gobiids (Tavolga 1956; Colombo *et al.* 1982). Of various internal body fluids from gravid females tested, only ovarian fluid and freshly extruded eggs elicited a courtship behaviour in frillfin gobies (Tavolga 1956). My results suggest that this may be true of round gobies, as homogenate from eggs and urogenital tissue elicited ventilatory and EOG (A. Belanger *pers. comm.*) responses in round goby males. After application of urogenital extracts, some of the male round gobies also responded to the odours by displaying a black, courtship colouration (*pers. obs.*).

I found that HCG and muscle tissue of a female injected with HCG did not cause the same increases in ventilation. Osmic male round gobies were tested with HCG injected, non-gravid, females in the winter. Since there were no increased ventilatory responses in the winter males exposed to gonadal extracts of winter females, this suggests that females were probably not producing pheromones at that time. Additionally, male round gobies responded significantly to E1 in the winter, implying that the olfactory ability of the male round goby is intact in the winter and that the chemical signals of the female are not present. It has been suggested that female round gobies track photoperiod and water temperature in order to anticipate breeding seasons (Moiseyeva and Rudenko 1976; Kulikova 1985). Males may track reproduction by responding to pheromones released by females when conditions are appropriate for reproduction. This suggests that male round gobies should have keen olfactory senses throughout the year in order to anticipate mating season and ensure propagation of their genetic diversity.

Seasonal changes to putative pheromones in female round gobies:

The round goby is polygynous. Several studies have shown that males will attract several females to a nest and guard eggs oviposited from several females in the spring and summer months (Miller 1984; Jude *et al.* 1992; MacInnis and Corkum 2000). Since female egg production seems to be regulated by temperature and photoperiod (Moiseyeva and Rudenko 1976; Kulikova 1985), and females are multiple spawners, males may synchronize their territoriality and nest-building with female reproductive development. Thus, pheromone communication should not be important to females during the winter

months because at that time, females should be conserving energy and maximizing growth and survivorship during the winter months. Eisthen *et al.* (2000) suggests that seasonal olfaction is controlled by gonadotropin releasing hormone (GnRH), so it would be expected that males and females would show an increased ventilatory response or would respond to lower concentrations of putative pheromones during breeding season compared to non-breeding season due to an increase in the excitability of OSNs. Partridge *et al.* (1976) also found that sexually mature males exhibited an increased sensitivity to food odour, suggesting that perhaps the increased response to a pheromone reflects a general increase in olfactory responsiveness induced by physiological changes associated with reproduction.

In *G. jozo*, males are known to release ETIO-g from the leydig cell rich, mesorchial gland (Colombo *et al.* 1982). Male round gobies have a similar gland, but it is referred to as the cement gland in this species. This gland may also be used for pheromonal production of ETIO or ETIO-g. It has been suggested by Murphy *et al.* (2001) that the pheromone released by male round gobies is probably the steroid ETIO as both males and females of this species respond with significant increases in ventilation and EOG responses. A close relative to the Gobiidae, the Cottidae, have reproductive behaviour comparable to that of gobies in that territorial males defend a nest-site to which females are attracted for spawning. Results from a combination of techniques (chromatographic characterization, behavioural bioassay, electrophysiological recording) have led Dmitrieva *et al.* (1986; 1988) to propose that sexual interactions of the yellowfin baikal sculpin (*Cottocomephorus grewingki*) are synchronized by the release of three

distinct male pheromones from the gonads, two of which are steroidal.

As in the black goby, steroid glucuronides seem to be important as attractants of female conspecifics released by males. In male African catfish (*Clarius gariepinus*) interstitial cells of the seminal vesicle and testis synthesize steroid glucuronides which attract ovulated females (van den Hurk and Resink 1992). EOG recordings demonstrated that each steroid in the steroid mixture was detected by the olfactory system of the African catfish with detection thresholds as low as 10^{-11}M (Resink *et al.* 1989b). Steroid glucuronides, produced in the testis, also induce ovulation in female zebrafish, *Brachydanio rerio* (van den Hurk *et al.* 1987; van den Hurk and Resink 1992).

In behavioural trials performed in the summer, female round gobies did not respond significantly to concentrations of 10^{-9} M ETIO. Although all female fish used for behavioural experiments in the summer were sexually mature (i.e. GSI ~ 10%), some may not have been in the proper reproductive condition to respond to the odours as only ovulated female *G. jozo* exhibit reproductive behaviour (attraction and oviposition) in response to ETIO-g (Colombo *et al.* 1982). This research suggests that it is necessary for a female round gobies to be ovulated in order to perceive pheromones, however the female reproductive condition is not always easy to discern. Also, data from male round gobies tested with extracts of gravid females suggests that most of the females used in the summer trials were reproductively mature and that females may control reproduction chemically. There have been no other studies that have shown that round goby females respond seasonally to odourants.

Summary:

Male and female round gobies respond more to putative pheromones, E1 and ETIO, when their GSI values are increased, compared to those with lower GSI values. This indicates that GSI is a good measure of sexual maturity and that sexual maturity may be related to olfaction. This study suggests that round gobies, males and females respond to odours seasonally, with an increase to putative pheromones in the summer time. My research shows that reproductive status has an affect on olfactory ability. Females round gobies did not show a response ETIO in the winter, however they showed a significant response during the reproductive season while males responded to smaller concentrations of putative pheromones in the summer.

Olfactory occlusion allowed me to show conclusively that increases in ventilation in response to putative pheromones and urogenital extracts are mediated through olfaction as those fish which had been occluded did not respond to putative pheromones or urogenital extracts. Ventilation rate as a bioassay for measuring responses to pheromones is a useful technique as this study shows that ventilation changes are due to a sensory event in the nose. The ventilation-reflex assay may be used to study responses mediated by olfaction in other fish species also containing accessory nasal sacs.

Female round gobies contain urogenital compounds that elicit a behaviour response in males. This extract may contain a pheromone that males recognize and track throughout the year. There is evidence to that female round gobies only produce this pheromone during breeding season. Thus the female round goby may use this pheromone to forcast favourable breeding conditions and her reproductive state to the male.

GENERAL DISCUSSION

Relationship between olfactory structure and responses to odours:

Structurally, the peripheral olfactory organ of the round goby is unique when compared to other teleosts (e.g., Yamamoto and Ueda 1979; Yamamoto 1982) in that the round gobies has dorsally located olfactory epithelium. This widespread distribution of olfactory epithelium in the olfactory chamber of the round goby is novel in teleosts and is reminiscent of amphibian (*Xenopus laevis*; Hansen *et al.* 1998) peripheral olfactory organ structure. The round goby has a centrally located longitudinal olfactory lamellae, a feature found only in benthic dwelling fish. This structure, and the two prominent accessory nasal sacs found in most fish in the order Perciformes, may increase water flow through the olfactory chamber, over the sensory epithelium (Burne 1909; Yamamoto 1982). These structures seem to be associated with the hydrodynamics of benthic-dwelling, slow moving fish such as the round goby.

The relationship between the olfactory system and ventilation is clear and direct. The presence of accessory nasal sacs suggests that the round goby regulates flow of odourant molecules over the sensory surface. When the round goby ventilates, the accessory nasal sacs are pumped to push water through the olfactory chamber and over the olfactory epithelium. This demonstrates that the increase in gill ventilation, during exposure to putative pheromones is triggered by olfactory sensory input. The olfaction-ventilation reflex is supported by the fact that anosmic fish, treated with $CuSO_4$ or nasal occlusion, do not respond to odours. Connection between gill movements and pumping

of the olfactory chamber has been observed in another fish as movement of water through the olfactory chamber of black rockfish, *Sebastodes melanops*, was brought about by the alternate compression and expansion of the accessory nasal sacs during normal respiration of the fish (Johnson and Brown 1962). Whether or not this response is voluntary or involuntary is unknown.

Olfaction appears to be important to gobies. In *Gobius niger*, the reaction to food extracts is vigorous (Pipping 1926; 1927), and in *Bathygobius sporator* sex discrimination and courtship behavior is mediated by olfaction (Tavolga 1956). The use of pheromonal communication may account for migratory behaviour in four riverine Hawaiian gobies, *Lentipes concolor*, *Stenogobius genivittatus*, *Awaous guamensis*, *Sycyopterus stimpsonir* (Sorensen 2001) and may be related to reproductive behaviour in *Gobius jozo* (Colombo *et al.* 1980). Physiological and behavioural responses to odorous molecules have been recorded from the round goby (Murphy *et al.* 2001). My study demonstrates that olfaction, enhanced by gill ventilation may be important for chemical communication.

Steroidal sex pheromones in the round goby:

This study, along with research by Murphy *et al.* (2001) strongly suggests that round gobies use pheromones to communicate their reproductive status. Most of the work conducted on female pheromonal communication has been performed on Cyprinids (e.g., *Carassius auratus*, *Carassius carassius*, and *Cyprinus carpio*) (Irvine and Sorensen 1993; Stacey *et al.* 1994a; Stacey *et al.* 1994b; Bjerselius *et al.* 1995). However, it has

also been shown that female fish, other than cyprinids produce chemical attractants and mating signals. Grandolfi (1969) showed that males of the guppy (*Poecilia reticulata*) are significantly attracted to the water in which conspecific female have been kept, but females conspecifics did not show an attraction to the water in which males have been kept. Spermiated male catfish (*Icalurus punctatus*) also are attracted to chemical substances released by a ripe female of the same species (Timms and Kleerkoper 1972). In a southern Asian anabantid fish *Colisa*, males build bubble-nests on the surface of the water. This nest building behaviour was found to be induced by a chemical substance emitted by the conspecific females (Rossi 1969). Nest building behaviours in male round gobies also may be induced by females that track biotic conditions important to mating and releasing pheromones when conditions are appropriate.

In the round goby, hormonal compounds that are released by females may have pheromonal function and appear to be different from strategies used by fish that employ a competition type mating system in which males compete directly for access to ovulated females (Emlen and Oring 1977). For example, the round goby does not detect prostaglandins commonly detected by many fish species (Stacey and Cardwell 1995), indicating that prostaglandins may not give pheromonal function in this species (Murphy *et al.* 2001). My study suggests that urogenital extracts contain pheromones, presumably estrogenic compounds (Speckler and Sullivan 1994) because they are received by a second individual of the same species and release a specific behaviour (Karlson and Lüscher 1959).

Summary:

My research is important because it is the first study to conclusively link olfaction with gill ventilation rates in a fish species by showing that fish that were sensory deprived could not respond to odours at any season of the year. The ventilation assay was first introduced in a study by Murphy *et al.* (2001), but they did not use fish that were sensory deprived to test their assumption that olfaction and gill ventilation were closely related. The link between olfaction and gill ventilation is also supported by the fact that male round gobies demonstrate a dose-response effect to putative pheromones.

My research was also the first study to show that female round goby urogenital extracts invoke a behavioural response in males, but this has been shown in another goby (Tavolga 1956). However, future research linking behavioural changes with olfactory ability in the round goby will have to be performed to examine the importance of chemoreception in the mating activities of the round goby. Although the findings suggest that the round goby has evolved a complex sex pheromone system, further research is required to determine the attraction, specificity, and chemical make-up of natural pheromones in the round goby. Such research is warranted because the goby pheromone system is the first to be explored in a perciform fish, and may serve as a new model system for paternal nest-guarding species. Also, further understanding of the round goby pheromonal system may help fisheries biologists find a natural and feasible approach to control the spread of the round goby in the Great Lakes and other aquatic ecosystems.

FUTURE WORK

It would be interesting to histologically examine the testes of the round goby to see if this species possesses leydig cells, which have been shown to release a hormonal pheromone, in the black goby (*Gobius jozo*). The male of the black goby released ETIO-g from the leydig cells to attract the females to a guarded nest. Prominent mesorchial glands with steroidogenic features have been described in other gobiids (Miller 1984). This finding is of particular interest in that it provides a rare example of a discrete gland apparently specialized for the production of pheromone. It would be interesting to know if ETIO-g attracts ripe female round gobies in a Y-maze, and to see if the source of these hormonal pheromones are the same as the round goby's family member, the black goby.

A comparison of OSN neuron quantity, dimorphism, and distribution in females and males during reproductive and non-reproductive season would be helpful. Also, it has been shown by Eisthen *et al.* (2000) that increases in GnRH leads to increased olfactory sensory neuron sensitivity (increased magnitude in inward currents) in mudpuppies (*Necturus maculosus*). GnRH distribution should be studied in round gobies during reproductive and non-reproductive seasons and then correlated with ventilatory responses to putative and natural pheromones.

APPENDIX A

RECIPES:

Stock Solutions:

Stock A= 27.6 g sodium phosphate dibasic (Na_2HPO_4) in 1L of distilled water
Stock B= 28.4 g sodium phosphate monobasic ($NaH_2PO_4 \bullet H_2O$) in 1L of distilled water

BUFFERS:

0.1 M Phosphate Buffer, pH=7.4:

460 mL of 0.2 M diphosphate buffer (stock A)
1540 mL of 0.2 M monophosphate buffer (stock B)
2000 mL of distilled water

0.2 M Phosphate Buffer, pH=7.4:

90 mL of stock A
30 mL of stock B

0.1 M Phosphate Buffered Saline (PBS), pH=7.4:

8.76 g NaCl
0.2 g KCl
Add to 1L of 0.1 M Phosphate buffer

Sucrose solutions:

10% sucrose - 1g of sucrose in 10 mL of distilled water
20% sucrose - 2g of sucrose in 10 mL of distilled water
30% sucrose - 3g of sucrose in 10 mL of distilled water

FIXATIVES:

4% paraformaldehyde:

4g of paraformaldehyde
100 mL of distilled water

1. Dissolved the paraformaldehyde in distilled water and heat to approximately 55°C.
2. Add a little NaOH to help dissolved the paraformaldehyde.
3. Adjust pH to 7.4 with either HCl or NaOH.

Zamboni's Fixative:

20 g of paraformaldehyde (2%)
850 mL of 0.1 M phosphate buffer, pH=7.4
150 mL of saturated picric acid (1.2%), **PICRIC ACID WILL EXPLODE IF HEATED!!**

1. Dissolve paraformaldehyde in buffer and heat to approximately 70°C.
2. Add a little NaOH to help dissolved the paraformaldehyde.
3. **COOL** the solution.
4. Add picric acid and adjust pH to 7.4 with either HCL or NaOH.

Karnovsky's Fixative (modified):

1. Prepare 0.56 M cacodylate buffer by dissolving 11.984 g sodium cacodylate in 100 mL of distilled water. pH to 7.5 with HCl.
2. Prepare 1.2% paraformadehyde by dissolving 0.6g of paraformadehyde in 50 mL of distilled water. Heat to approximately 55°C and add a bit of NaOH to facilitate dissolving. Put on ice to cool.
3. Prepare 2% gluteraldehyde by diluting 8 mL of 25% gluteraldehyde into 23 mL of distilled water.
4. Combine 25 mL of 0.56 cacodylate buffer with 50 mL of 1.2% paraformadehyde and with 25 mL of gluteraldehyde.
5. Add 0.02g of $CaCl_2$.

Gilson's Fixative:

1. Dilute 100 mL of 60% ethanol in 880 mL of water.
2. Combine with 15 mL of 80% nitric acid.
3. Add in 18 mL of glacial acetic acid.
4. Weight and add 20 g of mercuric chloride to this solution.

PREPARATION OF SEMI AND ULTRA THIN PLASTIC SECTIONS:

Medcast	80 mL
Dodecenyl succinic anhydride (DDSA)	50 mL
Nadic methyl anhydride (NMA)	45 mL

Combine all three chemicals and mix slowly, for approximately 1 hour. Add 1.75 mL of 2,4,6-tri[dimethylaminomethyl]phonel (DMP-30), drop by drop, while mixing. Mix for an additional 20 minutes slowly to avoid air bubbles. Freeze for long term storage. Before using make sure that plastic has completely dissolved.

Plastic tissue preparation:

1. Inject modified Karnovsky's fixative directly into the nostril of the anesthetized round goby. Dissect out the peripheral olfactory organ. Immerse fixed olfactory organs in Karnovsky's fixative overnight.
2. Tissue was trimmed and osmicated in 1% OsO_4 for 2 hours in an ice bucket covered with aluminum foil.
3. The tissue was dehydrated using ice cold ethanols (alcohols are stored in freezer before use).
   - 50% ethanol for 10 minutes on ice
   - 70% ethanol for 10 minutes on ice
   - 80% ethanol for 10 minutes on ice
   - 90% ethanol for 10 minutes on ice
   - 100% ethanol for 10 minutes on ice
   - 100% ethanol for 10 minutes on ice
   - 100% ethanol (absolute alcohol) for 10 minutes (3 x at room temperature)
5. Infiltrate with propylene oxide for 10 minutes (3 changes at room temperature).
6. Immerse tissue into a 3:1 mixture of propylene oxide:epoxy for 5 hours.
7. Incubate tissue in a 1:3 mixture of propylene oxide: epoxy overnight on a rotator.
8. The next day, place tissue in 100% epoxy and rotate overnight replacing with fresh epoxy 2 times.
9. Orient the tissue in embedding molds, pour in fresh 100% epoxy and place in a 70°C oven and bake overnight or until plastic hardens.

STAINS UTILIZED:

Toluidine Blue:

1. Dissolve 1g of sodium tetraborate in 100 mL of distilled water
2. Add 1g of toluidine blue, stir with stir bar
3. Filter before use with a syringe filter
4. Place slide on a hot plate on medium heat
5. Use syringe to apply stain on the section
6. Remove slide, once steam is observed and stain has just dried
7. Immerse slide in coplin jar containing distilled water
8. Allow slide to dry and coverslip with either Entellan or Permount

Uranyl acetate:

1. Prepare 7% uranyl acetate by dissolving 0.35g of uranyl acetate in 5 mL of 70% ethanol and stir. Cover the container since uranyl acetate is light sensitive. This may take some time.
2. Once the uranyl acetate is completely dissolved (should not be foggy, if it is, discard immediately), filter with 0.2 μm syringe filter.
3. Place filtered uranyl acetate into the wells of a porcelain staining dish, place a grid in each well.
4. Put the staining dish into a humidity chamber and incubate at 37°C for 30 minutes.
5. Allow to cool at room temperature for 10 minutes.
6. Remove each grid and place it through several changes of distilled water.
7. Allow grids to air dry within self closing forceps.

IMMUNOCYTOCHEMISTRY PROTOCOLS:

Fluorescence Microscopy:

1. Fix the tissue using Zamboni's fixative or 4% paraformaldehyde and immerse overnight.
2. Use whole tissue or sectioned peripheral olfactory organ using a cryostat (after cryoprotection).
3. Stain tissue using the following steps:

10 minutes in refrigerated 0.1 M PBS
10 minutes in refrigerated acetone
10 minutes in refrigerated 0.1 M PBS
15 minutes in blocker (goat serum)
Apply antibody (leave overnight)
10 minutes refrigerated PBS (3 washes)
Apply secondary antibody (leave for 1 hour)
10 minutes cold 0.1 M PBS (3 washes)
Coverslip with Vectashield®, sealing with clear nail polish

Avidin-Biotin Complex (ABC)/ Diaminobenzidine tetrahydrochloride (DAB) Staining:

1. Fix the tissue using Zamboni's fixative or 4% paraformaldyhyde and immerse overnight.
2. Use sectioned peripheral olfactory organ using a cryostat (after cryoprotection).
3. Stain tissue using the following steps:

10 minutes in cold 0.1 M PB
10 minutes in cold acetone
10 minutes in cold 0.1 M PB
Apply antibody (leave overnight)
10 minutes cold PB (3 washes)
Apply secondary antibody (leave for 1 hour)
10 minutes cold PB (3 washes)
Immerse sections in ABC reagent (30 min)
10 minutes cold PB (3 washes)
Apply DAB solution (watch under microscope for reaction)
10 minutes cold PB (3 washes)
95, 95, 100, 100% Ethanol solutions (40 s each)
50 : 50, ethanol : xylene (40 sec)
30 seconds in 100% xylene (3 washes)
Mount with Permount[c]

APPENDIX B

Calibration of behavioural chamber:

Table 3: The absorbance of coloured dyes at time intervals used in behavioural trials. This was done in order to investigate the distribution of odour and to calibrate the chamber.

Time (minutes)	In nest (OD)	Outside nest (OD)
4	0.047	0.029
7	0.044	0.042
10	0.037	0.038
15	0.047	0.045
20	0.043	0.041
25	0.039	0.039
30	0.042	0.039

Absorbance of thoroughly mixed tanks is 0.046 OD.

This dye study demonstrates that the colour, representing an odour, is similar to the thoroughly mixed tank in the nest at the times ventilation rates were measured.

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